American Journal of

Veterinary Research

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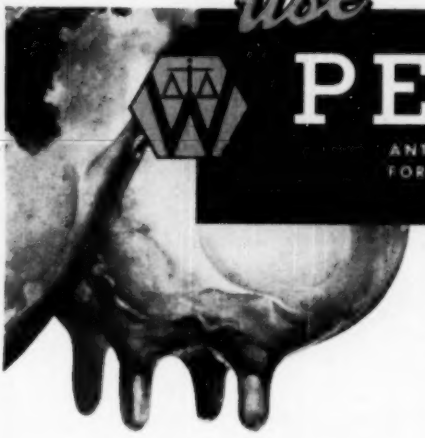

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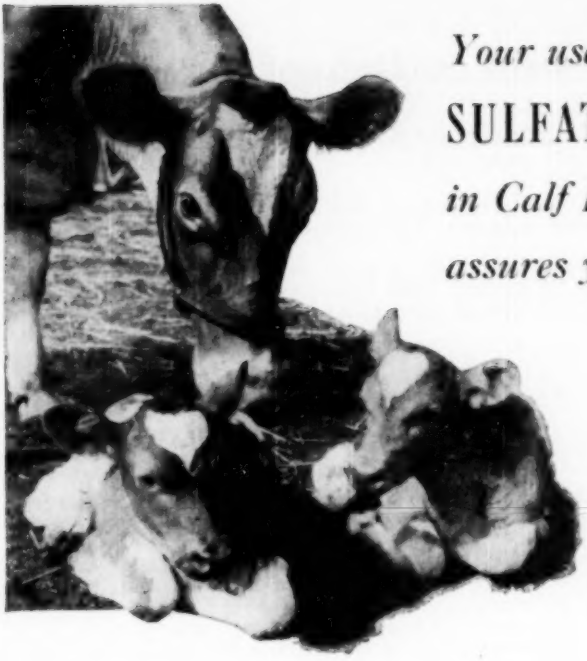

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The Response of Thyroidectomized and Intact Dogs to Thyroidal Stimulation

R. F. BORGMAN, D.V.M., M.S., and E. P. REINEKE, B.S., M.A., Ph.D.
East Lansing, Michigan

ALTHOUGH THYROID therapy is used occasionally in small animal practice, little experimental evidence has been available to determine the best level and sequence of dosage. Consequently, such therapy has been on an empirical basis up to the present time. Since more dogs are living past the stage of puppyhood diseases, geriatrics becomes important in small animal practice. Endocrine therapy is used in man at the present time to prolong useful existence. It has been demonstrated in man that the metabolic rate declines with age.[5] It is probable that the same trend of metabolism exists in dogs, and thus a place for thyrotherapy of senility is suggested. With the advent of less expensive sources of thyroid hormone, such as thyroprotein,[20] it becomes of interest to investigate the possibilities of thyroid therapy in the dog.

As the basal metabolic rate is a well-established measure of thyroid hormone activity, the measurement and expression of the metabolism assumes some importance. Unless one uses trained dogs, however, it is practically impossible to obtain a basal metabolic rate without the use of anesthetics. Hypnotic doses of amytal, barbital sodium, dial, ipral, neonal, and phenobarbital produce only a slight depression of the metabolic rate of human beings.[1,26] Doses of amytal sufficient to produce deep narcosis in dogs resulted in little change of the respiratory quotient and calories produced. The metabolism usually decreased less than 10 per cent even when the body temperature fell as much as 2 to 3 degrees.[8] Light dosages of dial (Ciba Pharmaceutical Co.), together with morphine, resulted in no significant change of metabolism in trained dogs receiving the test with and without anesthesia.[12] Schirmer,[23] in his work thus far, has found a small drop in the metabolic rate of trained dogs under light anesthesia with pentobarbital sodium.

Clinical metabolism values in human beings are conventionally expressed as calories per square meter of surface area. Although several formulas have been derived for the surface area of the dog, there is such variability among breeds and in results on the same dog that the authors believe the results should be related instead to a power of the body weight. Since different powers of the body weight have been used for the dog,[16,12] the authors chose to use the figure given by Brody[4] of the 0.73 power of the body weight.

It was the object of this research to form a basis for thyroid therapy in the dog. By administering exogenous thyroid to intact and thyroidectomized dogs and observing the body functions, especially the metabolic rate, it was possible to determine the therapeutic levels and tolerance levels of protamone* and desiccated thyroid.

The data reported herein are taken from a thesis submitted by Dr. Borgman to the School of Graduate Studies, Michigan State College, East Lansing, in partial fulfillment of the requirements for the degree of master of science in physiology, June, 1949, and are published with the approval of the director of the Agricultural Experiment Station as journal article No. 1065 (n.s.).

From the Department of Physiology and Pharmacology, Michigan State College, East Lansing.
Dr. Borgman is a fellow of the Research Council of the American Veterinary Medical Association.

Acknowledgment is made to the Ciba Pharmaceutical Co., Summit, N. J., for a research grant used to defray a part of the expenses of these investigations.

*Synthetic thyroprotein supplied by the Cerophyl Laboratories, Inc., 2438 Broadway, Kansas City, Mo.

GENERAL EXPERIMENTAL PROCEDURE

The basal metabolic rate determinations were all made at the same time at night, after the dogs had fasted for twelve hours. The room temperature was maintained at 24 to 26 C. To prepare the dogs for the metabolism determination, sodium pentobarbital** was administered intravenously until a light anesthesia was produced. The degree of anesthesia was judged by the point at which the pupil just began to contract and the dog did not resist pressure around the nose, indicating that he would allow fixation of the mask. The dog was allowed to rest for thirty minutes before the determination was begun. A modified Benedict-Roth type respirometer† was used with a rubber mask attachment to fit tightly when held over the muzzle. The determination was continued long enough to obtain a steady slope over a six-minute period. After the oxygen consumed was corrected to

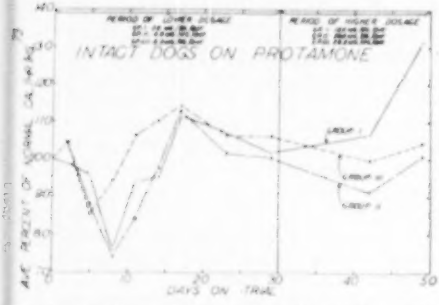

Fig. 1—The effects of protamone on the metabolic rate of three groups of intact dogs.

standard conditions, the calories per hour were obtained by multiplying the liters of oxygen consumed by the factor 4.825.[16] The specific metabolism, used to compare values in these experiments, was obtained[4] by the formula:

$$\text{Specific metabolism} = \frac{\text{Cal. per hr.}}{\text{Kg. body wt.}^{.75}}$$

The pulse rate, body weight, and rectal temperature were taken immediately before metabolism determinations. Readings taken after thirty minutes of anesthesia revealed no temperature decline. Dickinson's dog food‡ was fed *ad libitum* throughout the experiments.

**A 3 per cent solution of sodium pentobarbital containing 10 per cent ethyl alcohol was used.

†The bell used was about half the size used in the human apparatus, having a capacity of 12.6 cc. per millimeter of height.

‡Dickinson's Dog Food is marketed by Albert Dickinson Co., Chicago, Ill.

TRIALS WITH PROTAMONE IN INTACT DOGS

Trials were conducted on intact dogs to determine the tolerance level for protamone.

The experimental procedure was as follows. After normal values had been ascertained by three consecutive determinations over a period of one week, the dogs were divided into three groups and placed on protamone.

Group 1 consisted of 4 dogs, a female, 9-month-old Cocker-Pointer crossbred weighing 10.7 kg.;† a male, 9-month-old Collie weighing 11.6 kg.;** a female 5-year-old mongrel weighing 8.0 kg.; and a male 3-year-old Collie weighing 15.4 kg.

Group 2 consisted of 3 dogs, a female 9-month-old Cocker-Pointer crossbred weighing 10.6;† a male 9-month-old Collie weighing 15.3 kg.;** and a female 3-year-old Cocker Spaniel weighing 11.9 kg.

Group 3 consisted of 4 dogs, a male, 9-month-old Cocker-Pointer crossbred weighing 12.3 kg.; a female, 9-month-old Cocker-Pointer crossbred weighing 11.4 kg.;† a male, 9-month-old Collie weighing 16.7 kg.;** and a female, 5-year-old Cocker Spaniel weighing 10.9 kg.

The dosage of protamone was given in capsule form daily as follows:

	First thirty days	Next nineteen days
Group 1	2 mg./kg./day	12 mg./kg./day
Group 2	4 mg./kg./day	20 mg./kg./day
Group 3	8 mg./kg./day	28 mg./kg./day

RESULTS

On the lower dosages administered for the first thirty days, no apparent symptoms of hyperthyroidism were observed. It was impossible to keep food records, but the food consumption appeared to have risen. The condition of the 5-year-old mongrel in group 1 improved. Previous to the trial, he was lethargic and had a rough coat. During the trial, this dog showed more vigor and an improved coat. The 9-month-old Collie in group 3 had intermittent diarrhea and appeared to become slightly overexcitable.

On the higher dosages administered for the next nineteen days, symptoms of hyperthyroidism began to appear. Differences were not evident by groups but more by breeds. The Cocker-Pointer crossbreds in all groups were more tense and exhibited a strained expression about the face. The Cocker Spaniels, the 5-year-old mongrel in group 1, and the 3-year-old Collie in group 1 showed no hyperthyroid symptoms. The Collie litter mates in all groups became hy-

†Cocker-Pointer crossbred litter mates.
**Collie litter mates.

perexcitable and the 9-month-old Collie in group 3 had a persistent diarrhea and roughened his coat on the cage. His general appearance became that of an older dog.

The metabolism, body weight, pulse rate, and body temperature did not come into

TABLE I—The Development of Tetany in Dogs Thyroidectomized with Two Parathyroids Left *in Situ*

	Previously untreated	On protamone Group 1	Group 2	Group 3
No. dogs in the group	5	4	3	4
No. dogs developing tetany	0	2	2	4
Ave. days for tetany to develop	0	3	3	2.8
No. which succumbed	0	1	2	3
No. which survived	5	3	1	1

equilibrium during the trial. The metabolism records for all groups followed the same trend and there was little differentiation in the response (fig. 1). All groups showed a preliminary drop in the metabolic rate in determinations taken on the eighth day of the trial. The fall in metabolic rate occurred in all dogs except the 5-year-old mongrel in group 1 and ranged down to 54 per cent of the normal metabolism. On metabolism determinations taken on the seventeenth day after the start of dosage, the average rise in metabolism for all groups was 10 to 15 per cent above normal. For the last thirteen days on the lower dosage, the metabolic rates of the groups declined toward normal.

On the higher dosages of protamone, the metabolic rates of groups 2 and 3 were about normal. Group 1 showed a rise, but this was due to a marked rise in the metabolic rate of 1 dog.

The pulse rate could not be correlated with the metabolic rate but was generally above normal when the dogs were given protamone. The increase in average pulse rates for the groups on the lower dosages of protamone ranged from 1 to 47 per cent. Group 3 had the greatest rise in pulse rate. On the higher dosages of protamone, there was a 13 to 25 per cent increase in pulse rate, groups 2 and 3 being the highest.

The body temperatures were about 1 per cent above their previous normal in all groups on protamone. There was no differentiation between groups.

TRIALS WITH DESICCATED THYROID IN INTACT DOGS

The object of trying desiccated thyroid on intact dogs was to see if the results were similar to those obtained with protamone.

Two dogs were used, a 7-year-old male mongrel weighing 9 kg., and a 5-year-old male Scottish Terrier weighing 8 kg. Daily dosages of 50 per cent stronger than U.S.P. desiccated thyroid were administered for twenty-eight days at the rate of 4 mg. per kilogram of body weight per day to the mongrel and 2 mg. per kilogram of body weight per day to the Scottish Terrier. Three metabolism determinations were made to ascertain the normal values over a period of two weeks. There were no visible effects upon the dogs' behavior while on trial. The metabolism determination on the seventh day after the start of trial showed a drop in the metabolic rate to 67 to 70 per cent of normal. The subsequent values were near normal, with the body weight showing a slight decline. The body temperature remained near normal, while the pulse rate showed a marked rise in both dogs (fig. 2).

EFFECTS OF THYROIDECTOMY

Because normal, intact dogs were found to have a high resistance to exogenous thyroid, it was thought that thyroidectomized animals

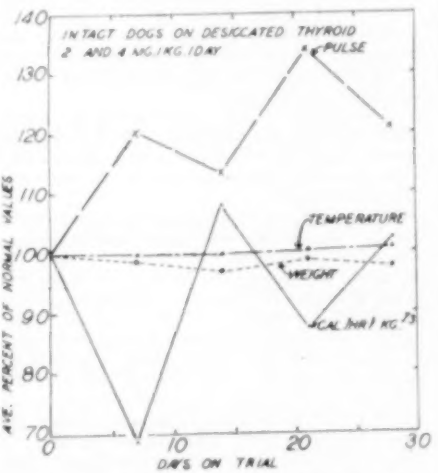

Fig. 2—The effects of 50 per cent stronger than U.S.P. desiccated thyroid upon the body functions of 2 intact dogs. Note the marked rise of pulse rate while the metabolic rate rises little above normal.

would be more suitable for use in establishing therapeutic dosage levels.

The thyroidectomy was performed aseptically and consisted of the complete removal of the thyroid glands, while the two lateral parathyroids and their blood supply were dissected from the thyroid tissue and left in place.

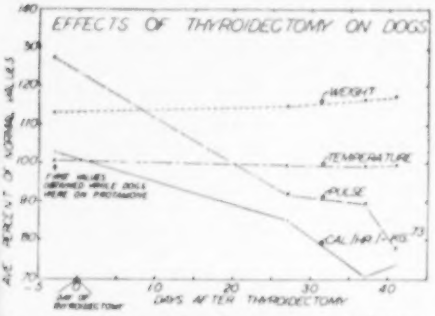

Fig. 3—The effects of thyroidectomy on the body functions of the dog. The increase in body weight was due mostly to increased fat. The metabolism values are expressed as calories per hour per kilogram of body weight.[25]

Thus, the dogs had two of the four parathyroids removed, and the blood supply to the remaining two was injured. In a few cases, one or both of the lateral parathyroids had to be removed with the thyroid gland and then reinserted into muscle tissue. Later autopsy revealed these parathyroids to be functioning. No thyroid gland proper was found in any of the carcasses at autopsy. The surgery was performed on the three groups of dogs receiving protamone about two to three days after cessation of dosage.

The weights of the thyroid glands showed a decrease as the previous dosage of protamone increased. However, these observations were not clear cut, probably due to the varying breeds used.

The development of tetany is shown in table 1. It may be seen that dogs previously untreated developed no tetany. Since Dr. Borgman performed all the surgery, there was little difference in respect to impairment of the parathyroid function. There was no difference in the extent of tetany when one or both of the parathyroids were taken out and inserted into muscle. The greater the previous dosage of protamone, the greater the number of dogs developing tetany, the more rapid the onset of tetany, the greater the severity of tetany, and the greater the mortality. Therapy with parenteral calcium gluconate, parathyroid extract, and calcium chloride given in the drinking water did little to improve the dogs. Since accessory thyroid tissue is common in dogs,[25] a radioactive iodine tracer method was used to detect any remaining thyroid tissue in the thyroidectomized dogs. The method has been worked out by Reinhardt.[22] As the thyroid gland has great affinity for circulating iodine in the blood stream, a tracer dose of radioactive iodine (I° 131) may be injected intravenously and its location detected with a Geiger-Müller counter. If a dog is entirely free of thyroid tissue, the radioactive iodine will be quickly secreted through the kidneys and intestines. If there is thyroid tissue, the radioactive iodine will be concentrated there and the count over the rest of the body will be high because of the formation of thyroid hormone which retains the radioactive iodine in the body. Thus, accessory thyroid tissue can be found by locating points of radioactivity and a maintenance of high counts over the body when a Geiger-Müller counter is used twenty-four to forty-eight hours after injection of radioactive iodine.

Evidence of accessory thyroid tissue was found in 2 dogs. These dogs also failed to show any hypothyroid symptoms such as lethargy, fall in metabolic rate, fall in pulse rate, fall in the body temperature, or increase in body weight. Geiger-Müller counts in the live animal and after autopsy indicated accessory thyroid tissue to be present in the thymus and aortic regions in one and the aortic region near the kidney in the other.

The symptoms of thyroidectomy are summarized in table 2 and figure 3. The figures are based on 3 dogs which previously received protamone and for which there were no records of normal metabolism. Other symptoms observed in all the thyroidectomized dogs thirty-five to forty days after thyroidectomy, except those with indications of accessory thyroid tissue, were lethargy, loss of appetite, and a slight edema of the under parts. Light anesthesia for metabolism determinations required less sodium pentobarbital. In 1 case, the edema was present in the face also.

TABLE 2—The Effects of Thyroidectomy on Some of the Body Functions of Dogs

	Normal	Average after thyroidectomy	Per cent of normal values
Cal./hr./kg.[25] (metabolic rate)	4.714	3.402	72.2
Body weight (kg.)	13.000	15.200	117.0
Pulse rate/min.	86.800	72.600	83.8
Body temperature (degrees F.)	100.000	99.500	99.5

The body weight was 113.1% of normal at the time of thyroidectomy.

TRIALS WITH PROTAMONE AND DESICCATED THYROID IN THYROIDECTOMIZED DOGS

The objective was to ascertain the amount

of protamone and desiccated thyroid which would return the body functions of thyroidectomized dogs back to normal. The amount of each thyroid preparation which would do this is considered to be the therapeutic dose.

After two metabolism determinations were made to find the thyroidectomy values, the dogs were divided into two groups. The protamone group included 3 dogs: a 1-year-old female Cocker-Pointer crossbred weighing 12.6 kg.; a 1-year-old male Smooth-Haired Fox Terrier weighing 9.9 kg.; and a 1-year-old female short-haired mongrel weighing 9.7 kg. The desiccated thyroid group included 4 dogs: a 2-year-old female Smooth-Haired Fox Terrier weighing 9.5 kg.; a 1½-year-old male Gordon Setter weighing 19.5 kg.; a 1-year-old male long-haired mongrel weighing 13.3 kg.; and a 2-year-old female Cocker Spaniel weighing 13.9 kg.

The dosage is given herewith:

Dosage of Protamone for Protamone Group

First 14 days	Next 22 days	Last 14 days
0.5 mg./kg./day	1.0 mg./kg./day	2.0 mg./kg./day

Dosage of Desiccated Thyroid for Desiccated Thyroid Group

2.0 mg./kg./day	4.0 mg./kg./day	8.0 mg./kg./day

The thyroidectomy symptoms of edema, lethargy, and loss of appetite began to disappear after one week on dosage. By two weeks, the symptoms of edema were gone and the dogs became more lively. After three weeks on trial, all dogs were lively and eating well; at this time they had been on the immediate dose level of exogenous thyroid for one week. The protamone group recovered from the thyroidectomy symptoms more rapidly than the desiccated thyroid group. The results on other body functions are summarized in table 3 and figure 4.

There was the expected rise in metabolic rate and body temperature. The body weight also increased during dosage, probably because of increased food intake. The pulse rate was raised in the desiccated thyroid group, while in the protamone group it remained practically normal.

Since thyroidectomy resulted in a 27.8 per cent fall in the metabolic rate, the metabolism would have to increase to 138.5 per cent of the thyroidectomy level to be back at normal. Because of the small numbers of dogs used, these figures are relative. It required 1 mg. per kilogram per day of protamone and 4 to 8 mg. per kilogram per day of desiccated thyroid (50% stronger than U.S.P.) to raise the metabolism back to the normal level (fig. 5).

The protamone was four to eight times stronger than the desiccated thyroid used here as determined by the assay on thyroidectomized dogs. If the highest dosages of both preparations are compared, 2 mg. per kilogram of body weight per day of protamone increased the metabolic rate to 132.2 per cent of the thyroidectomy level, while 8 mg. of thyroid per kilogram of body weight per day raised the metabolic rate to 131.3 per cent. Thus, protamone appears four times more potent than desiccated thyroid (50% stronger than U.S.P.). If the 1-mg. per kilogram per day dose of protamone which raised the metabolism 140.8 per cent is compared with the 8-mg. per kilogram per day dose of desiccated thyroid which caused a 131.3 per cent rise, protamone appears to possess eight times the potency of this desiccated thyroid.

Another method of comparison makes use of the 0.73 power of the body weight by the following formula:

$$\text{Per cent rise in metabolism} + \frac{\text{Kg. body wt.}^{0.73}}{\text{Mg. thyroid dosage}} = \text{A constant.}$$

The constants were averaged for each dosage. The averages for each group were added and compared. Protamone appeared 5.1 times as potent as desiccated thyroid (50% stronger than U.S.P.).

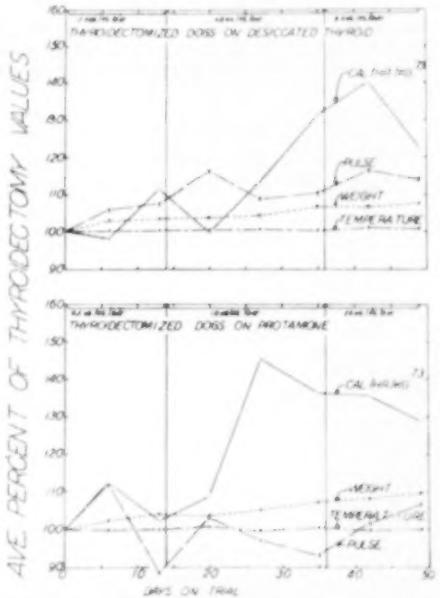

Fig. 4—The effects of desiccated thyroid and protamone on some body functions of thyroidectomized dogs.

The thyroxine content of the protamone used, as determined by the highly specific isotope-dilution method [6] was 0.578 per cent. The desiccated thyroid contained 0.3 per cent iodine, of which approximately 25 per cent would be thyroxine iodine. This would be equivalent to 0.1147 per cent thyroxine. Thus, the protamone contained 5.04 times as much thyroxine as the desiccated thyroid, a ratio that agrees very well with the figure of 5.1 obtained from the comparative metabolic effects. By this estimation, protamone would be about 7.5 times the strength of desiccated thyroid U.S.P.

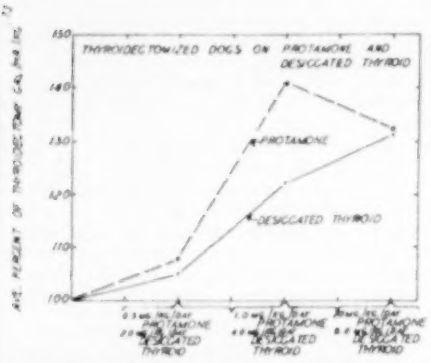

Fig. 5—The effects of protamone and desiccated thyroid on the metabolic rate of dogs. The metabolism values were averaged after the exogenous thyroid had time to take effect.

DISCUSSION

From the results of these experiments, it is evident that the dog has a broad tolerance range to thyroidal stimulation. Our results in this respect are similar to those of Drill and Hays[10] and Drill and Shaffer,[11] who showed that dogs will tolerate large amounts of thyroid without losing weight if adequate amount of B vitamins are given. Similarly, Danowski[7] reported only a transient weight loss in dogs fed large amounts of thyroid. Consequently, it appears that in dogs receiving an adequate diet and with reasonable precautions on dosage, thyroid therapy can be undertaken where indicated clinically with little risk of overstimulation.

The much greater cardio-accelerating action of thyroid than of protamone that was observed in our experiments is of particular interest in view of the reports that desiccated thyroid contains a "cardiotrophic" factor that has a stimulating action on the heart that is separate from its metabolic stimulation.[17,18,19] Inasmuch as excessive heart stimulation is highly undesirable in therapeutic use, protamone has a distinct advantage over thyroid in this respect.

So far as the authors are aware, the aggravation of hypoparathyroid tetany by previous conditioning with protamone has not been reported previously. The fact that this should occur is not surprising, however, since it has been demonstrated that during hyperthyroidism the rise in calcium excretion is greater than the increase in metabolic rate.[2,15]

The body temperature followed the trend expected. That is, the body temperature rose after the administration of thyroid as

TABLE 3—The Effects of Protamone and Desiccated Thyroid on Body Functions of Thyroidectomized Dogs*

1) Lowet dose range	Protamone 0.5 mg./kg./day	Desiccated thyroid 2.0 mg./kg./day
	Metabolism as per cent of thyroidectomy level	
Cal./hr./kg.$^{.75}$	107.7	105.0
Body weight	103.1	103.2
Pulse rate	99.8	106.7
Body temperature	99.9	100.2
2) Medium dose range	Protamone 1.0 mg./kg./day	Desiccated thyroid 4.0 mg./kg./day
Cal./hr./kg.$^{.75}$	140.8	122.2
Body weight	108.1	105.6
Pulse rate	95.3	109.4
Body temperature	100.0	100.4
3) Higher dose range	Protamone 2.0 mg./kg./day	Desiccated thyroid 8.0 mg./kg./day
Cal./hr./kg.$^{.75}$	132.2	131.3
Body weight	109.0	107.1
Pulse rate	104.2	115.0
Body temperature	100.0	100.7

*Metabolism expressed as cal./hr./kg.$^{.75}$

found by Dott[9] and Drill and Hays.[10] The drop in body temperature after thyroidectomy confirms the reports of other work on thyroidectomized dogs.[9,3]

The transient decline in the metabolic rate of intact dogs on the seventh to eighth day of dosage with either protamone or desiccated thyroid appears to be similar to the decline in the metabolism of hyperthyroid human beings given desiccated thyroid.[6] This phenomenon cannot be fully interpreted in the light of present knowledge. However, it probably reflects a change in hormone equilibrium in which a new balance is established between the thyroxine supply and its inactivation by the system. It has been shown[14] that thyroxine inactivation by the liver is increased during hyperthyroidism; there is also increased excretion of an antimetabolite in the urine.[13] The thyroid gland itself may be involved in this mechanism since thyroidectomized dogs and myxedematous human patients[25] show a larger metabolic rise for a given thyroid dosage than do normal subjects.

The normal metabolic rates of the dogs as determined in this study were within the range reported in Brody's compilations[5] and the work of Galvao.[12] The average values were somewhat higher than the constant of 70.5 calories per day per kilogram $^{0.73}$ given by Brody,[4] however. The largest deviations were seen in the smaller, more energetic breeds.

Summary and Conclusions

1) Doses of 1 mg. of protamone per kilogram of body weight per day and 4 to 8 mg. per kilogram per day of 50 per cent stronger than U.S.P. desiccated thyroid were required to raise the metabolism of thyroidectomized dogs back to the normal range. Thus, the therapeutic range may be estimated as follows: 1 to 2 mg. per kilogram per day of protamone and 6 to 12 mg. per kilogram per day of desiccated thyroid U.S.P.

2) Adult dogs were able to tolerate doses up to 8 mg. of protamone per kilogram per day for a month without demonstrable hyperthyroid symptoms.

3) Protamone had four to eight times the potency of desiccated thyroid (50% stronger than U.S.P.) as assayed by calorigenic effects on thyroidectomized dogs. The most probable ratio between the two is 5 : 1.

4) Protamone and desiccated thyroid (50% stronger than U.S.P.) both caused a transient drop in the metabolic rate on the seventh to eighth day of dosage. Large doses of up to 28 mg. of protamone per kilogram per day did not cause a marked rise in the metabolic rate of normal dogs.

5) The protamone antagonized the action of the parathyroid gland, causing tetany in dogs thyroidectomized, with two of the parathyroids removed, while under the influence of protamone. The incidence of tetany was proportional to the previous dosage of protamone.

6) Replacement therapy with protamone and desiccated thyroid alleviated the symptoms of thyroidectomy.

7) Thyroidectomy resulted in a 27.8 per cent drop in the metabolic rate and a decline in pulse rate and body temperature. The body weight increased subsequent to thyroidectomy probably because of increased deposition of body fat.

8) Desiccated thyroid had a greater effect upon the pulse rate relative to its metabolic effect than did protamone.

9) The body weights of dogs increased when they received moderate daily amounts of protamone.

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Studies of the Virus of Equine Infectious Anemia

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and A. L. BRUECKNER, V.M.D.
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ELECTRON micrographs were prepared which showed bodies assumed to be the virus of equine infectious anemia. For these studies, virus was furnished by Dr. C. D. Stein of the Pathological Division, Bureau of Animal Industry, Washington, D. C. This was collected by him from horse 1401 of the 1947 New Hampshire outbreak and was sent to us in the form of lyophilized serum.

This lyophilized serum was diluted with physiologic saline to an approximate 10 per cent suspension, and 0.5 cc. of this suspension was injected subcutaneously into a horse. On the thirteenth day after inoculation, the temperature reached 103.8 F. The animal was bled on the fourteenth and sixteenth days after inoculation, at which time the temperature was 104.0 F. and 106.0 F., respectively. The horse was moribund at the time of the second bleeding. The serum was passed through a Seitz filter and stored at –60 C. This filtered serum was injected into a horse and produced the characteristic symptoms of infectious anemia.

For electron microscope studies, this serum, as well as filtered serum samples from several normal horses, was subjected to centrifugation at 52,000 r.p.m. for three hours in a Spinco ultracentrifuge, with a temperature change of approximately 1 degree. The sediment from each of these serums was resuspended in 1.0 cc. of distilled water, and a small drop of this suspension was placed on parlodion film supports. Excess fluid was removed with a fine capillary pipette, the films were dried, and then were shadowed with 20 mg. of chromium at tangent 1/8. Examination was in an RCA electron microscope (type EMU).

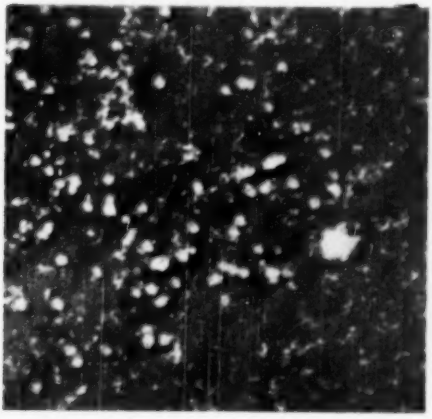

Fig. 1—Swamp fever virus from horse serum. x 41,000 diameter. Shadowed with 20 mg. of chromium at tangent 1/8.

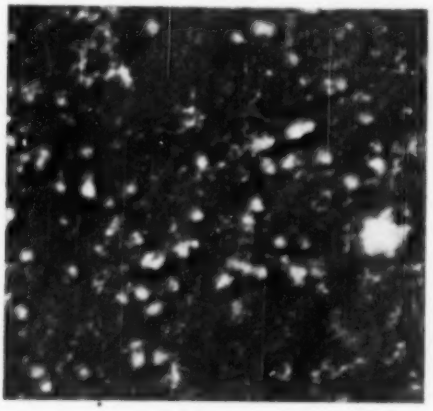

Fig. 2—Swamp fever virus from horse serum. x 56,000 diameter. Shadowed at tangent 1/8 with 30 mg. of chromium.

Material prepared from serum from the infected horse showed bodies of variable size, some with indistinct outline, but almost all of round shape, as in figures 1 and 2. Serum from the normal horses showed none of these bodies. By direct measurement of the electron micrographs, these bodies varied from 11 $m\mu$ to 59 $m\mu$. The mean diameter was 30 $m\mu$.

Balozet,[1] who carried out filtration experiments with the equine infectious ane-

From the Live Stock Sanitary Service Laboratory, University of Maryland, College Park.

Presented before the Section on Research, Eighty-sixth Annual Meeting, American Veterinary Medical Association, Detroit, July 11-16, 1949.

mia virus, using collodion membranes of different porosity, reported that the size of the virus particles is probably between 18 and 50 mμ.

In a paper[2] presented to the U. S. Livestock Sanitary Association meeting in Denver, Colo., in October, 1948, the authors reported on the establishment of the virus of equine infectious anemia in rabbits by intravenous injection of citrated blood. The virus had been passed at the time of that report through eight serial passages. Not all injected rabbits showed an increase in temperature. Some showed only one period of temperature elevation lasting for periods varying from twenty-four hours to as long as a week. Other rabbits showed recurring high temperatures during observation. Rabbit virus from the third serial passage, when injected into a horse, produced a chronic form of disease in the animal, characterized by intermittent periods of elevated body temperature similar to the natural disease in that species. The first significant temperature rise occurred as early as the sixth day after injection or as late as the thirty-third day, with successful further passage. On the average, the temperature was first elevated at the end of two weeks. There was only a slight tendency for increase in the percentage of rabbits in any passage which showed evidence of infection by temperature rise. No other noticeable symptoms appeared, although, in the later passages, engorgement of the spleen and liver was more frequently noted at autopsy.

Rabbit passage was continued through the twentieth subinoculation.

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DISCUSSION

CHAIRMAN SCHALM: This paper is open for discussion. Are there any questions relative to Dr. Brueckner's paper?

DR. CHARLES L. DAVIS (Colorado): Have histologic examinations been made of the livers of these rabbits to compare the lesions with those found in the horse?

DR. BRUECKNER: We have done very little, Dr. Davis. On one occasion, however, we sent some liver material from 1 of these rabbits into Dr. H. W. Schoening's department, and they reported no lesions such as are seen in the liver of the horse which dies of infectious anemia. The pathology and histology must be studied more carefully than it has been to date.

The Effect of Homogenization, Pasteurization, and Lyophilization on Egg-Yolk Sodium Citrate Diluents for Bull Semen

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EGG-YOLK diluents are the most popular and the most successful solutions used in the preservation of bull semen. The increase in size of breeding establishments has rendered the daily preparation of a diluent a tedious and expensive task.

In the preparation of a diluent containing egg yolk, it has been the common practice to equalize the proportion of egg yolk to citrate or phosphate solution. Swanson,[1] however, found in the preparation of a semen diluent using egg yolk and buffer that the proportion of egg yolk to buffer need not be in exactly equal parts for optimum semen livability. It has been observed at the Iowa Experiment Station, Ames, Iowa, that, when the proportion of egg yolk is lowered below one half, the solution becomes less viscous, tending to run out of the inseminating pipette and separating from the citrate solution when stored in vials or test tubes. Although Phillips[2] and Phillips and Lardy[3] used fresh egg yolk (minus the vitelline membrane) and a phosphate-buffered solution, and Salisbury[4] later used an egg-yolk and sodium citrate diluent, their research did not include extensive use of dried egg yolk or pasteurized egg yolk.

This experiment was designed to (1) develop an egg-yolk sodium citrate diluent in which the egg yolk would not settle out when the proportions of egg yolk were not exactly one half of the diluent; (2) prepare a diluent free from bacterial contamination; and (3) prepare a diluent capable of being stored under refrigeration for considerable time.

TABLE 1—The Effect of Homogenized, Lyophilized Egg-Yolk Diluent on Motility and Survival Time of Five Ejaculates from 5 Bulls*

Mediums	Bull No.	Hours									
		24	48	72	96	120	144	168	192	216	240
Undiluted semen.	2840	2	3	4	5	5	D				
	2814	2	2	4	4	5	D				
	3082	2	3	4	4	4	5				
	3050	2	3	3	4	4	5	5	D		
	2881	2	3	5	5	D					
Semen + fresh buffered egg-yolk diluent. Dilution 1 : 70.	2840	1	2	2	2	3	4	5	5	5	D
	2814	1	1	2	2	3	3	4	4	5	5
	3082	2	2	3	3	4	4	5	5	5	D
	3050	1	1	1	2	2	3	3	4	4	5
	2881	1	2	2	3	3	4	4	5	5	5
Semen + homogenized, lyophilized, egg-yolk buffered diluent. Dilution 1 : 70.	2840	1	2	2	2	3	4	5	5	5	D
	2814	2	2	3	3	3	4	4	5	5	D
	3082	1	1	2	2	2	4	4	4	5	5
	3050	1	2	3	3	3	4	4	4	5	5
	2881	1	1	2	2	3	3	4	4	5	5

*Motility graded 1 to 5 with 1 indicating greatest motility.
D = dead.

EXPERIMENTAL PROCEDURE

Experiment 1.—Fresh egg yolks with the vitelline membrane included were homogenized and lyophilized. The homogenization was accomplished by the use of a small hand homogenizer. The lyophilization (drying under vacuum at very low temperature) was performed with a laboratory-size machine. Sodium citrate and sulfanilamide were combined with the egg yolks in amounts to make a 3 per cent solution of sodium citrate and a 0.3 per cent solution of sulfanilamide before the yolks were

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From the Department of Dairy Husbandry, Iowa State College, Ames.

processed. After lyophilization, distilled water was added to make a mixture of 3 parts egg yolk to 5 parts water. All of the egg yolk would not reconstitute, thus leaving a sticky mass on top of the mixture, even after attempts to incorporate it with an electric mixer. Sterile cheesecloth (mesh 44 x 40) was used to separate the material. The filtrate, which measured 78 per cent of the original volume, was used as a diluent for five ejaculates from 5 different bulls. The average motility is recorded in table 1.

Four additional samples of the lyophilized product were prepared and stored in the refrigerator at 40 F. for thirty days. This material was then reconstituted and filtered. The livability of sperm diluted with this material was equal to that of sperm diluted with the fresh reconstituted material.

Although it was impossible to reconstitute all of the material after lyophilization, the results obtained indicate that egg yolk can be prepared in this manner, stored under refrigeration, and reconstituted at a later date as needed. The results show that, due to homogenization, egg yolk will not separate from the citrate solution.

thirty minutes, with constant agitation, and stored at 40 F. in sterile, stoppered bottles. Bacterial cultures taken at intervals during the storage period did not indicate any contamination.

The homogenized, pasteurized material did not settle out when standing and maintained motility of sperm comparable to that of fresh egg yolk. A portion of this pasteurized diluent was stored in a refrigerator for thirty days and used with satisfactory results at the end of this period.

Table 2 indicates survival time and motility of five ejaculates.

CONCLUSIONS

1) Homogenization prevented settling out of egg yolk in egg-yolk citrate diluent up to dilutions of 3 parts egg yolk to 5 parts citrate solution.

2) Lyophilized buffered egg yolk may be reconstituted and used as a semen diluent. Complete reconstitution of the lyophilized product was not possible.

3) Pasteurized egg-yolk sodium citrate diluent stored under refrigeration offers a sterile diluent that can be prepared in large quantities and used as needed.

TABLE 2—The Effect of Homogenized, Pasteurized Egg-Yolk Diluent on Motility and Survival Time of Five Ejaculates from 5 Bulls*

Mediums	Bull No.	Hours									
		24	48	72	96	120	144	168	192	216	240
Undiluted semen.	2840	2	3	4	4	5	D				
	2814	2	3	4	D						
	2082	2	2	4	4	5	D				
	3050	2	3	4	5	5	D				
	2881	2	4	5	D						
Semen + fresh egg-yolk citrate diluent. Dilution 1 : 70.	2840	1	2	2	2	4	5	5	5	D	
	2814	2	2	2	3	3	4	4	5	5	D
	2082	2	2	3	4	4	5	5	5	D	
	3050	1	2	3	3	3	4	4	5	5	5
	2881	2	2	3	3	4	4	4	5	5	D
Semen + homogenized, pasteurized, egg-yolk citrate diluent. Dilution 1 : 70	2840	1	2	2	2	3	4	5	5	5	5
	2814	2	2	2	3	3	4	4	5	5	5
	2082	1	2	2	2	3	3	3	4	4	5
	3050	2	2	3	3	3	4	4	5	5	D
	2881	1	2	3	3	4	4	5	5	5	D

*Motility graded 1 to 5 with 1 indicating greatest motility.
D = dead.

Experiment 2.—The considerable handling required in reconstitution of lyophilized egg yolk into a buffered diluent greatly increases chances of bacterial contamination. Accordingly, an attempt was made, using egg yolk, to prepare a sterile diluent that could be kept for some time under refrigeration in a form ready to use.

An egg-yolk citrate diluent was prepared by adding 3 parts of homogenized egg yolk to 5 parts of a 3 per cent sodium citrate solution containing 0.3 per cent sulfanilamide. This material was then pasteurized at 143 F. for

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The Excretion of Steroid Hormones Concerned with Controlling Reproductive Processes in Animals

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ALTHOUGH THERE are a number of recent reviews of the steroid hormones,[9,20,21,25,41,58,59,64,69] none has been devoted specifically, as is the present paper, to the excretion of steroids in animals.

It is important to know the rate at which hormones are secreted under the various reproductive states for diagnostic and therapeutic purposes. One means of ascertaining the secretory rate is to determine the rate at which the hormones or their derivatives are eliminated in the excreta. Other methods involve anatomic or physiologic responses to a specific hormone, responses obtained through parabiotic union, or determining the amount of hormone in the secreting gland or in the blood.

The excretory rate method for determining secretory activity is limited by the fact that the hormone may be eliminated from the body in a biologically inactive form, thus necessitating chemical methods of quantitation. If the hormone is excreted in a conjugated form, the chemical treatment necessary for splitting the conjugates may result in molecular changes and further complicate the evaluation of results. In this regard, Gallagher[25] states, "No procedure is at present known which will cleave the conjugates of all steroids without any alteration of the steroid molecule."

Now, let us consider the excretion of the individual steroid hormones concerned with controlling reproductive processes in animals.

ESTROGENS

Rodents.—The relatively small amount of urine voided by rodents has made this group of animals unpopular as subjects for estrogen excretion studies. No chemical methods are available for the quantitative detection of low concentrations of estrogens. Talbot *et al.*[72] suggested a method, but Bender and Wilson[4] found it inaccurate. The latter workers compared the Talbot method with the biologic assay using rat urine and found 3μg. from an equivalent of 300 rat days of urine by biologic estimation as compared to 3μg. per rat per day expressed as estrone by the chemical method of assay—a difference of 300 times.

Beerstecher[7] studied the excretion of estrogen in the rabbit using the biologic assay method. He reported a higher estrogen excretion in summer than in winter and believed the low winter excretion was related to the period of anestrus during certain winter months. He found no rise in estrogen level of the urine at the time of ovulation as has been reported for the human being.[76] From a level of 2 to 4 rat units (r. u.) per liter in nonpregnant rabbit urine, the concentration rose to over 100 r. u. per liter at the sixteenth to twentieth days of pregnancy. During late pregnancy, the values fall, as in the human female and the mare. The concentration during pseudopregnancy followed a similar curve, but the actual values were only one-half that of pregnancy.

The Cow.—All authors agree that the estrogenic activity of cow urine is low as compared to the urine of the mare or to that of the human female. Aschheim and Zondek[3] were apparently the first to recognize estrogen in the urine of cows. By extracting urine with olive oil without previous hydrolysis, Turner *et al.*[75] found 11 r. u. per liter in nonpregnant cow urine, but Nibler and Turner[60] found none in one sample of urine. During the first one hundred days of pregnancy, the estrogenic activity of cow urine is very low; a steady increase from this time to term has been reported by all studying the problem.[1,29,42,43,62,65,75] The daily excretion values near the end of pregnancy reported by these workers varied from as low as 500 r. u.[65] to 6,000 r. u.,[29] when urine was extracted without hydrolysis. The peak excretion reported on hydrolyzed urine was 17,000 mouse units (m. u.) per liter.[1] Turner *et al.*[75] reported that Holstein-Friesian cows excrete more hormone than Jerseys, and dairy breeds more than the beef breeds. A rapid fall in the estrogen level of the urine at parturition[29] suggests a placental source of the hormone during pregnancy.

Levin[43] has studied the excretion of estrogen in the feces of cows and found 5,000 to 8,000 r. u. per kilogram of solids in late pregnancy. His studies indicated that estradiol accounted for most of the estrogenic activity. Levin postulated that estrone may have been converted to estradiol by microflora within the large intestine. Mamoli[46] has shown that such a conversion can be made with certain strains of yeast *in vitro*. On ordinary diets, the cow will eliminate about 3 kg. of solids in the feces daily and, thus, approximately 15,000 r. u. would be eliminated by this source. Assuming that the urine contains 2,000 r.u. per liter and

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that 10 liters are voided daily, the total amount eliminated in the urine would be comparable to the amount excreted in the feces. This calculation is based on free estrogen for the urine and total estrogen for the feces; thus, the amount excreted in the urine probably exceeds that eliminated in the feces. Siebke and Schuschania[60] reported that the total excretion of estrogens in women is divided almost equally between urine and feces.

The Horse.—Steroid excretion in the urine of the mare has received more attention than any other species with the exception of the human female because of the fact that the urine of the pregnant mare has served as an important source of estrogen for clinical use. This has made it possible to study steroids in residues of very large samples of urine after estrogen extraction.

Only one paper, to my knowledge, is concerned with the excretion of estrogen in the nonpregnant mare.[46] They conclude that there are two peaks of urinary excretion—one during estrus and another between the tenth and fifteenth days of the cycle. This conclusion hardly seems warranted on the basis of the data presented. Although they did not study the relationship of estrogen excretion to the estrous cycle, Glud *et al.*[47] give 5,000 m. u. per liter as the highest amount they found in the urine of the nonpregnant mare. Kust[42] reported large amounts of estrogen in the urine during estrus.

Month of pregnancy	*Milligrams of estrone per liter*
1	trace
2	0.7
3	5.5
4	33.0
5	84.0
6	80.0
7	104.0
8	104.0
9	46.0
10	19.0
11	4.9

Zondek[48] discovered that the urine of pregnant mares was a rich source of estrogens. Since this discovery, the estrogens in mare pregnancy urine have been the subject of much study but, in spite of this fact, there are only a few studies on the curve of excretion throughout pregnancy. The results of most workers studying the excretory rate are in good agreement.[8, 16, 39, 51, 56, 60, 47]

Using colorimetric estimation, Beall and Edson[8] expressed their figures, as given below, in terms of estrogen calculated as estrone, but their figures do not represent the total estrogenic activity, as 20 to 30 per cent of estrogenic activity was discarded in the strong phenolic fraction.

Kust[42] is the only one who has indicated that the estrogenic concentration rises steadily to the time of parturition, most authors showing a curve of excretion corresponding to the figures given above. Girard *et al.*[26, 27, 28] found three new estrogens in pregnant mare urine: equilin, equilenin, and hippulin. Wintersteiner *et al.*[78] isolated dihydroequilenin. Cartland and Meyer[11] obtained 59 mg. of equilin from 24 gal. of mare urine collected during the last two months of pregnancy. Using Beall and Edson's figures for total estrogen, it appears that equilin accounts for less than 5 per cent of the total estrogen in mare pregnancy urine. Girard[27] stated that the proportion of different estrogens varies tremendously at different stages of pregnancy. Hirschmann and Wintersteiner[37] isolated two estrogenic diols, 17-dihydroequilenin and β-estradiol from the urine of pregnant mares.

It appears that the placenta is the source of estrogen in the urine of the pregnant mare. Newton[45] reviewed the subject, and thus cursory mention of the evidence will suffice. Amounts of hormone within the normal range were excreted by a mare ovariectomized during pregnancy.[46] More hormone was excreted postoperatively than could be accounted for in the body as estimated by studies on the estrogenic potency of various body tissues of other mares sacrificed during pregnancy.[16] The first voided urine of the foal and the mare after parturition contains estrogen but within twenty-four hours it is absent from both.[39] This rapid disappearance from the urine of the foal would suggest that the fetal gonads, which histologic studies had indicated might be a likely source,[15] were not secreting the hormone. Since the fetal placenta is the one tissue separated from both the foal and the mare at parturition, the evidence points strongly to it as the source of the estrogen.

The presence of large amounts of estrogen in the urine of the stallion is an anomaly.[40] Deulofeu and Ferrari[16] obtained only 1.5 mg. of estrone per liter. This low figure is possibly explained on the basis that most of the estrogen in stallion urine is estradiol.[41] Haussler[35] reported great variations in the amount of estrogen in stallion's urine, 35,000 to 300,000 m.u. per liter. Cartland *et al.*[12] found values ranging from 0 to 38,000 r. u. per liter. No doubt, future research will show that age and breed determine to a considerable extent the great variation which is reported.

PROGESTERONE

No evidence is available to indicate that progesterone is ever excreted in an active form. This means that the excretory form of the substance must be recognized by chemical means

in order to obtain information on its rate of excretion by the excretory method. Apparently, the excretory form varies in different species and this has complicated the problem.

A review of the literature has not revealed a single paper dealing with a systematic study of the excretion of progesterone derivatives in various reproductive states of any animal. This is in contrast to the abundant work on the excretion of pregnanediol in the human being, not only during various reproductive states but also under different pathologic conditions. Because of this dearth of studies on progesterone excretion in animals, there is no need to dwell on the subject at length.

Marker[49] reported 25 mg. of pregnanediol –3(∝)–20(∝), 15 mg. of allopregnanediol –3(∝)–20(∝), and 3 mg. of allopregnanediol –3(β)–20(∝) per gallon of urine from pregnant cows. Stevenson[71] was unable to recover any pregnanediol from either pregnant cow or mare urine, but this is probably explained by the fact that a method adapted for human urine was employed. Casady, in our laboratory, encountered similar difficulties using methods developed for human urine. Bull's urine contains about four times as much pregnanediol as cow urine and, in fact, is the richest source known.[52] The different pregnanediols appear in the same relative proportion as in the urine of pregnant cows and women. Steer urine, on the other hand, is devoid of pregnanediol. Marker[49] stated, in this connection, "The absence of the pregnanediols from steer's urine is in marked contrast to the large amounts present in bull's urine and suggests that these steroids are formed in the testes, or by the influence of the testes on some other gland; . . ."

Pregnanediol appears to be absent from sow pregnancy urine but contains pregnanol –3(∝) –one–20 and allopregnanol–3(β)–one–20.[58]

The urine of pregnant mares contains pregnanediol,[52] but the proportions of the different pregnanediols are very different from those obtained from human pregnancy urine.[71] Other than those steroids previously mentioned, pregnanedione, allopregnanedione, allopregnanol–3(β)–one–20, and uranol–11–one–3 have been recovered from mare pregnancy urine.

Pregnanediol has been reported to be absent in the urine of the chimpanzee,[11] Rhesus monkey,[54] and in the urine of pregnant rabbits and cats.[58]

Atkinson and Hooker[3] and Atkinson and Leathem[4] have studied progesterone secretion during pregnancy, pseudopregnancy, and lactation in the mouse, not by the excretion method, but rather by noting specific morphologic responses to the hormone in the uterus. Presumably, the responses are not influenced by the presence of estrogens. This method will, no doubt, assume considerable importance in those species in which the excretory method is not adaptable.

Androgens

Androgens, or their derivatives in the urine, must be liberated for either chemical or biologic estimation.[25] The urinary androgenic content of most animals which have been studied is very low. Gustavson[26] gave the following concentrations in international units (I.U.) per liter: stallion, 8; bull, less than 1; ram, 4; and rat, less than 1. These values compare with 30 to 100 I.U. per liter in the human being, the values in men and women being similar. With this very low titer, it is not surprising that little work has been done on the subject. Whitten[77] reports the excretion of 5 to 19 mg. of neutral ketosteroids per day in pregnant ewes.

Green *et al.*[20] have found significant variations in androgen content of the urine from different lines of inbred boars using both biologic and chemical assay procedures. In one line, the average excretion per forty-eight hours was 2,122 γ calculated as androsterone as compared to 4,523 γ in another line. Similar differences were likewise observed by the use of biologic assay.

Studies on rabbits[11] and on monkeys[58] indicate that the removal of the ovaries has little influence on androgen excretion in the urine. Dorfman *et al.*[12] found that, after both gonadectomy and adrenalectomy in monkeys of either sex, they still continued to excrete about one third of the preoperative level of 17-ketosteroids.

Androgenic Activity of Cow Feces.—In 1942, Riley and Hammond[46] made the interesting observation that the feces of cows demonstrated androgenic activity when incorporated in the diet of day-old chicks. Chloroform and alcoholic extracts of the feces were likewise active; a chloroform extract of 1 Gm. of feces had at least the equivalent potency of 16 μg. of testosterone acetate. Longwell and Gassner[44] reported that the active substance is slightly soluble in chloroform and completely soluble in ethanol. Fractionation studies by these authors indicated that the active substance is in the neutral ketonic fraction. Riley and Hammond[46] reported that bull feces are lacking in androgenic activity; Gassner and Longwell[44] stated that bull and steer feces are "relatively inactive."

The androgenic activity of feces from cows in various reproductive states has been studied. Turner[74] reported that the excretion of androgen is highest a few days before parturition and during the first month of lactation. Gassner and Longwell,[44] on the other hand, stated the maximum potency is found during the last week of pregnancy and then drops sharply to zero at calving. Casady *et al.*[10] found low androgenic titers in the feces of cystic ovary cows. It is interesting that the cystic ovary cow assumes certain masculine characteristics and, like the bull, has little androgen in the feces.

Some attention has been directed to the question of the source of fecal androgens. Hammond[11] and Turner[12] found no androgenic activity in rumen contents and the latter author likewise found no activity in the contents of the small or large intestine. The significance of these results is obscured by the fact that the feces of the cows used in these studies were not tested. In other words, it is possible that the feces of the animals used were likewise negative.

Casady[13] studied some factors modifying fecal androgen excretion. The nature of the diet was found to have a profound effect upon the amount of androgen in the feces; three to four times as much androgen was excreted when cows were placed on a roughage diet (alfalfa hay and straw or alfalfa hay alone) as on a diet of concentrates alone. The androgenic potency per gram of feces was greater in most instances on roughage and the total feces voided daily was several times greater. Ovariectomy did not depress the level of fecal androgen, rather, a slight increase occurred. In the light of this finding, it becomes difficult to explain why the injection of equine gonadotrophin produced an apparent increase in two of three trials. A pronounced increase in androgen excretion occurred after unilateral adrenalectomy. It is possible that stress associated with the operation and with an infection during the postoperative period may have stimulated the remaining adrenal to increased function. It should be recalled, however, that Dorfman *et al.*[47] did not find a complete cessation of 17-ketosteroid excretion when both the gonads and the adrenals were removed.

Evidence at hand indicates that the androgenic activity of cow feces is readily destroyed. Turner[12] showed that the drying of feces at 65 C. or above resulted in a decreased potency. Gassner and Longwell[28] likewise reported that fecal androgens are heat labile. Jaap and Thayer[29] found no androgenic activity in sun-dried feces, whereas, Rubin and Bird[30] reported as much androgenic substance in sun-dried as in oven-dried feces.

How much androgen is excreted in the feces? Until the specific androgens in cow feces are known, attempts to express excretion in absolute terms are of little value. By comparing the chick comb response to feces with that of dehydroandrosterone, Casady *et al.*[31] calculated the daily excretion of an equivalent of 212 mg. of dehydroandrosterone on a diet of concentrates and 1,280 mg. on a roughage diet. Gassner and Longwell[28] found that 1 kg. of feces had an androgenic potency equivalent to 300 mg. of methyl testosterone or 180 mg. of androstenedione.

Further investigations are necessary before the full significance of the presence of androgens in cow feces is known. Nonetheless, the fact that there are differences in excretion dependent upon diet, sex, and pathologic states (such as cystic ovaries) justifies further study of the problem.

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Studies on the Subgross Anatomy of the Bovine Liver
II. The Pathology of Telangiectasis as Demonstrated by the Vinylite-Corrosion Technique

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THE LESION of the bovine liver commonly termed telangiectasis was apparently first reported by McFadyean in 1890.[1] The publications of Getty[2] and Pavcek *et al.*[3] give comprehensive lists of works dealing with telangiectasis. An average of 12,658,918 bovine carcasses were submitted annually to federal inspection in the years 1942 through 1946. Of this number, a yearly average of 240,709 (1.9%) of the livers were condemned because of telangiectasis.[4] The economic value of this loss has been adequately described by Getty.[2]

This report deals with the gross, subgross, and microscopic pathology of telangiectasis of the bovine liver, as seen in the San Francisco Bay region. The paper relates the gross appearance of telangiectasis with microscopic findings, and locates the lesion in relation to the anatomy of the entire liver. It is believed that these considerations will aid in arriving at a better understanding of the pathogenesis of telangiectasis.

METHOD OF PROCEDURE

The apparent incidence of telangiectasis in the San Francisco Bay region is low as compared to other packing areas of the country.[4] In spite of this fact, a sufficient number of pathologic livers were obtained during the period of observation to give a representative sample of the condition as it is found in this locality. The livers used were a portion of some 700 livers examined at a local packing plant at intervals between October, 1946, and July, 1947. Those subjected to vascular injection or histologic study had been condemned by federal meat inspectors; most of them because of telangiectasis.

Livers showing lesions in the caudate lobe were chosen for the subgross studies because of the comparative ease of producing satisfactory corrosion casts of that portion of the organ. It has been shown, in a previous publication, that the structure of the caudate lobe of the bovine liver may be taken as an index of the structure of the intact organ.[5]

In the making of a vinylite-corrosion cast, vinylite plastic fills any hollow structure, such as a blood vessel, and this represents the lumen of that structure. The cylindric masses of vinylite of a vascular injection represent the lumens of the vessels, the walls and surrounding tissues having been digested by the strong acid utilized in the technique. Telangiectasis, which is characterized by cavernous vascular spaces in the intact liver, is represented by bulbous masses of vinylite in the cast. These represent the lumens of the cavernous blood spaces. Therefore, whereas a histologic section indicates the extent of a lesion in only two dimensions, the vinylite cast, by clearly distinguishing telangiectatic vessels from apparently normal ones, allows the investigator to study the lesion in the third dimension.

Cellular changes were studied by means of sections prepared for microscopic observation and stained by a routine hematoxylin and eosin method. When it was deemed advantageous, special staining techniques were employed, consisting of Pollak's trichrome method, Best's carmine technique for demonstration of glycogen, and sudan IV for the demonstration of fat in frozen sections.[6,7] Sections stained with Giemsa's stain were studied in attempts to demonstrate organisms in the involved tissues. Tissues were subjected to fixation in less than thirty minutes after the death of the animal.

RESULTS

Gross Observations at the Time of Slaughter.—The lesions were seen as dark, purplish red spots in localized areas, or in marked cases, scattered throughout the substance of the organ. Lesions in the internal portions of the gland were approximately spherical in shape, or were seen as half spheres when located at the surface. The

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size of the lesions varied from 1 mm. to 2 cm. in diameter. The capsule of the liver was seen to be depressed over the larger spherical lesions. The large lesions, when sliced, displayed a slightly pitted appearance, due to the dilated vascular spaces in their structures. Only in a few instances were the tissues immediately surrounding the telangiectatic lesions grossly affected. In these isolated cases, the liver tissue appeared slightly lighter than the major portion of the gland. No lesions considered to be sawdust condition were observed during the periods in which the samples were collected.

Subgross Observations.—When a telangiectatic liver is injected in the manner described for normal livers,[5] the resultant cast will disclose rounded, multilobular masses of plastic, which represent the lumens of the dilated vessels comprising the lesion of telangiectasis (fig. 1). If a plastic of contrasting color is used for each of the three vascular systems of the liver, that system in which the lesion is found may be determined. A total of 130 lesions were tabulated from studies of portions of 11 livers condemned because of telangiectasis. All of these lesions occurred in the hepatic portal system. In no case, were lesions found in systems other than the hepatic portal. Since the sinusoids were not filled by the injection mediums, there was no possibility of the lesions being filled by a return flow of plastic introduced into a vascular system other than the hepatic portal.

There is a direct relationship between the total portion of a branch of the portal system involved in a given lesion and the volume of liver tissue contributing to the formation of the lesion. By determining the major branches of the hepatic portal vein and locating interlobular vessels, it has been possible to establish a method of designation of a given vessel by comparing its size with that of the interlobular vein and primary branch of the portal vein. The smallest lesions observed involved the inter-

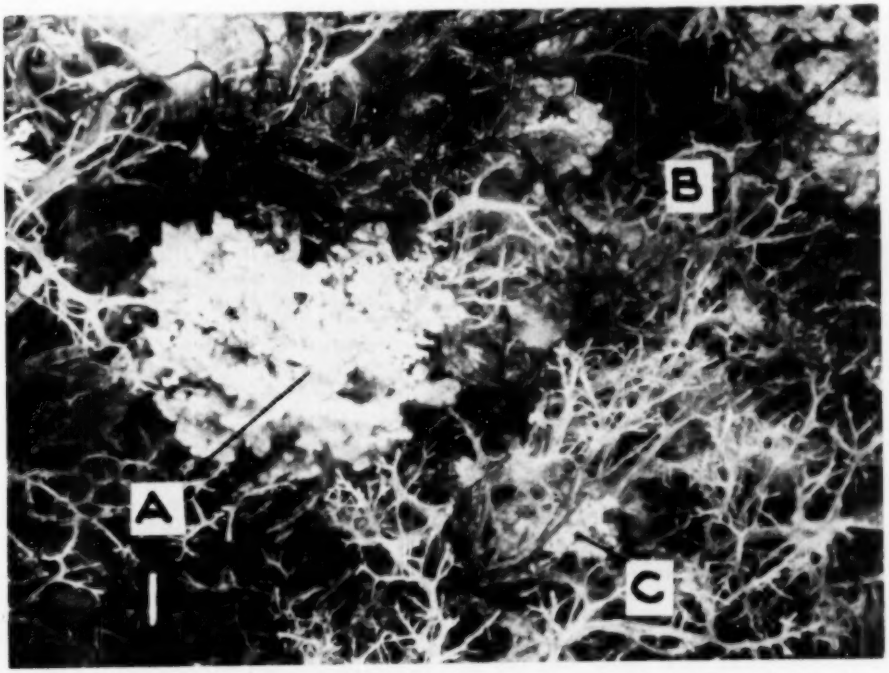


Fig. 1—Enlarged photograph of a vascular injection of the caudate lobe of a bovine liver showing a large lesion (A) and smaller lesions (B and C) in the hepatic portal system.

lobular branches of the portal system and vessels of slightly larger diameter. The larger lesions were seen in vessels as large as the third order of size. This statement is not intended to suggest that a lesion involving a third order vessel involves only that vessel. Vessels of this magnitude were seen to be involved only in larger lesions and, in these lesions, the majority of the vessels of the parent branch smaller than the third order were affected. Thus, the smallest lesions involved interlobular branches of the portal system, and the largest ones affected interlobular branches and all vessels of the portal system up to the designated order of size. Histologic studies reveal the sinusoids to be affected. Since the sinusoids are not demonstrated in corrosion specimens, the space occupied by involved sinusoids must be added to the space demonstrated in the cast in consideration of the total area involved by a given lesion. Whether this graded series of sizes of lesions and magnitudes of vessels indicates a progressive nature in the formation of the lesion cannot be stated at this time.

An occasional lesion may be found which has two major contributing branches of the hepatic portal system. This situation suggests the presence of the lesion in what has been termed a "hepatic portal circuit," which is a connection of one hepatic portal radicle with another, forming a potential collateral loop.[5] A second condition in which a single lesion has more than one portal vein entering it may occur as a result of coalescence of lesions involving radicles of the branch in question into a single large lesion (fig. 2).

Evidences of the parenchymatous origins of the degenerative changes leading to telangiectasis were disclosed by study of the vinylite casts of telangiectatic livers. These casts disclosed, in three dimensions, the rather limited areas of degeneration within which the lesion of telangiectasis forms, and its influence upon all vessels (portal) entering the region. An occasional normal vessel of the smaller orders of size may be seen, extending from the bulbous masses of a telangiectatic lesion (fig. 3, C). These small, normal branches from a telangiectatic-affected vessel are apparently outside of the area contributing to the formation of telangiectasis and therefore are not dilated as are the vessels existing within the telangiectatic region. Near, and yet clearly separated from, a well-formed telangiectasis lesion, an occasional small vessel may be seen which is affected by telangiectasis (fig. 3, B). These small vessels, when traced back to their origins, are completely separated from the vessels directly involved in the major lesion, and yet of the same common vein supplying the particular area of the liver. The small lesion represented by one or two dilated vessels is very close to the large lesion and no other terminal branches of the vessel are involved. These small vessels have come under the influence of the factors which have produced marked telangiectatic changes in the adjacent vessels.

In the description of gross observations, it was stated that telangiectasis may often involve localized areas. Corrosion casts of livers showing areas of telangiectasis disclose that such lesions arise from clusters of vessels originating from one or two of the larger branches of the portal vein supplying the particular region. Thus, a single second order vein may, in the course of its

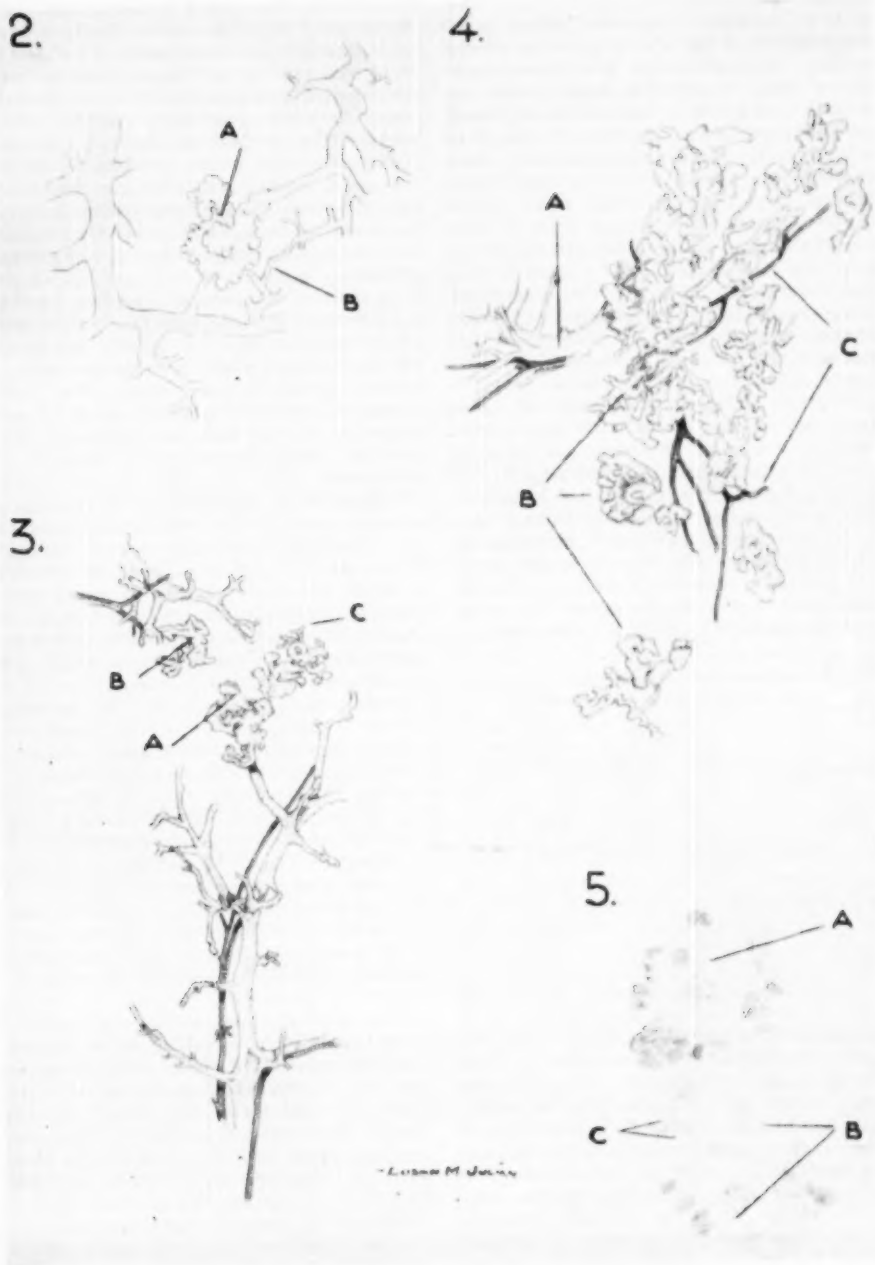
Legends for Illustrations on Opposite Page

Fig. 2—A single telangiectatic lesion (A) formed by two branches of the hepatic portal system. The lesion either formed in a hepatic portal circuit, as illustrated (B), or was the result of coalescence of two smaller lesions. (Diagrammatic sketch.)

Fig. 3—Influence of an area of tissue damage upon the radicles of the hepatic portal system. Lesion of telangiectasis (A). Small, affected radicle of hepatic portal vein (B) showing no direct physical connection with the larger lesion, yet demonstrating that it had been influenced by the same region of tissue degeneration which had caused the more prominent lesion (A). Small, unaffected radicle of hepatic portal vein extending from area of parenchymatous damage (C). (Drawing.)

Fig. 4—Detailed drawing of a portion of the large lesion of telangiectasis seen in figure 1, demonstrating that the pathology is limited to the hepatic portal venous system. Note normal branches of hepatic artery coursing through the lesion. Hepatic portal vein (A). Telangiectatic lesion in hepatic portal venous system (B). Branches of hepatic artery (C). (Drawing.)

Fig. 5—Drawing of hepatic parenchymatous cells undergoing a type of "swollen cell" degeneration. Swollen hepatic epithelial cell within which may be seen a red blood cell (A). Note manner in which the cytoplasm has accommodated itself to the intruding blood cell, demonstrating that this is not an artifact. Hepatic epithelial cells showing disturbed continuity of the cell membrane (lower cell) (B). Extruded nucleus and cellular débris (C). (Drawing.)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ramifications in a given region, contribute to the formation of several lesions, each separated from the others by spaces of apparently normal vessels and parenchyma. The vessels of other systems located in or near telangiectatic lesions are unaffected by the telangiectatic process. Figure 4 is a sketch of a large telangiectatic mass through which unaltered branches of the hepatic artery are coursing.

Microscopic Observations.—The microscopic pathology of the telangiectatic livers consisted of degeneration of hepatic epithelial tissue with a dilation of the intervening blood spaces. In all cases observed, the basic change was the same—a decrease in the parenchymatous tissue volume with a corresponding increase in blood spaces.

Necrosis of hepatic epithelial cells was a consistent finding; however, it was not the major finding in all cases. A type of "swollen cell" degeneration was seen to contribute considerably to the destruction of the liver architecture. This change consisted of a marked swelling of the cytoplasm of the epithelial cells, with final rupture of the cell membrane and the loss of the cell contents into the blood stream. The swelling was apparently due to a diffuse increase

TABLE I—Tabulation of Incidence of Telangiectasis and Sawdust in Three Packing Areas

	Percentage incidence		Proportional incidence	
1942-1946(a)	Sawdust	Telangiectasis	Sawdust	to telangiectasis
Omaha	3.230	1.30	2.5	- 1.0
Chicago	0.630	2.20	0.57	- 1.0
San Francisco	0.040	0.70	0.06	- 1.0
San Francisco local source Nov., 1946-Jan., 1948 (58,000 animals)	0.027	0.42	0.06	- 1.0

of intracellular fluid. Swollen, unruptured cells showed a rather even distribution of cytoplasmic material throughout the cell body. No droplet formation could be demonstrated, and experiments with selective stains (for glycogen and fat) failed to indicate the nature of the swelling medium. In one instance, a section made at the point of rupture of the cell membrane disclosed the cytoplasm exuding from the opening. Some of the sections disclosed red blood cells situated within the cell membrane and collected about the nucleus. Apparently, the red cells had entered through a break in the cell membrane. The orientation of the cytoplasmic fragments to the enclosed red cell indicated that the red cell was actually within the liver cell, and that the situation was not due to technical procedures (fig. 5). The nuclei of the swollen cells showed no visible changes. Normal nuclei, practically free of cytoplasm, could be seen within the blood spaces, as though being washed away from the scene of degeneration. Such a process could account for large losses of liver parenchyma.

In most of the sections, portions of the hepatic cords could be seen free within the larger veins of the liver. Usually, cytoplasmic and nuclear changes could be demonstrated in the isolated cords. The free segments apparently were the result of destruction of liver cells at two points, the free cells being the intervening portion of the hepatic cord.

Inflammatory cells were present in telangiectatic lesions with considerable regularity. The quantity of cells was not marked. They usually consisted of a mixture of lymphocytes, monocytes, and polymorphonuclear leucocytes; however, polymorphonuclear leucocytes were seldom the predominent cell type. Red blood cells in the dilated spaces appeared to be normal.

Study of a large number of sections shows a variable condition of the lining endothelium of the blood spaces. In lesions undergoing active degeneration, the endothelium is destroyed. In entire lesions, or in portions of large lesions showing little epithelial destruction, the endothelium is prominent. These facts suggest to the observer that the lesions studied were in several states of formation; those showing no endothelium, representing the more active stages of degeneration; those exhibiting well-lined blood spaces, representing the "organized" lesion.

Two sets of serial sections of telangiectatic lesions were of particular interest in that they exhibited fibrosis of the telangiectatic lesion. The sections showed that the liver was simultaneously infected with flukes. Much greater activity of the polymorphonuclear leucocytes was seen in these sections. The presence of fibrosis in these lesions is ascribed to the more prominent inflammatory changes of the concurrent infection. No fibrosis was noted in uncomplicated telangiectatic lesions.

During the period of observation and collection of specimens, the high incidence of telangiectatic-like lesions in the livers, condemned because of fluke infection, was noted in one lot of animals. These lesions were small and somewhat scattered throughout the tissue surrounding the gross lesions of fluke infection. Histologic study disclosed that the lesions, while having the general characteristics of telangiectasis, differed in predominantly exhibiting coagulation necrosis of the surrounding hepatic tissue. Furthermore, the blood of the dilated spaces was not normal, as in the case of a typical telangiectatic lesion. Staining reactions and cellular outlines were altered in such a manner as to indicate stagnation of the blood. Vessels of the hepatic canals located within areas of marked inflammatory reaction were seen to be sclerosed. Their lumens were completely obliterated. The histologic character of the lesions described differed sufficiently from those of livers uncomplicated by other pathologic changes that it is considered by the observer to be distinct from the uncomplicated telangiectasis.

In reviewing the results of subgross and microscopic studies, it appears that whatever the cause or causes of telangiectasis, the lesion arises as a result of parenchymatous changes. The circumscribed nature of the lesions, as indicated by study of the corrosion casts, is further supported by histologic study.

Discussion

Jaeger,[5] in 1907, laid the anatomic foundation for the formation of telangiectasis in the bovine liver. According to his studies, telangiectasis results primarily from the breakdown of liver cells with dilation of the intervening blood spaces. The present report and the recent work of Getty[2] are in agreement with this point of view.

Within the liver, there exists an internal equilibrium of pressures of the liver parenchyma and the circulating blood. The distribution of the vascular system of the liver is such that, within almost any portion of the organ, the pressure of the blood supports the tissue, and the compressional strength of the tissue supports the blood spaces. Particularly is the latter true in the areas about the sinusoids. Should either the pressure of the blood or tissues of a given area increase, it must increase at the expense of the other factor. Should either decrease, the relative space of the other is increased. In the development of a telangiectatic lesion, the loss of epithelial tissue results in increased space for the blood, hence the lesion should be expected in a system exhibiting positive pressure and whose walls are most easily dilated. The studies reported in this paper have demonstrated that telangiectasis occurs consistently in the hepatic portal system, as would be expected according to this hypothesis (*see* fig. 4).

Telangiectasis arises as a passive dilation of the hepatic portal vein. The toxic agents might be distributed by the hepatic portal artery or any other route, but, due to the relatively weak walls of the hepatic portal vein, the vein dilates in response to the decrease in tissue pressure resulting from epithelial degeneration. Telangiectasis may be a manifestation of a variety of pathologic conditions. In the San Francisco Bay area, it arises as a result of the loss of hepatic epithelial tissue due to primarily degenerative changes. Getty,[2] at Omaha, Neb., and Chicago, found telangiectasis to be the result of primarily inflammatory changes. At the present state of knowledge concerning the etiology or etiologies of telangiectasis, it is well not to think of the lesion as a disease entity, but rather as a pathologic process of the bovine liver of unknown cause or causes.

The studies here presented throw doubt upon existing theories of the correlation of telangiectasis and sawdust condition as expressed by Getty,[2] and by Jensen, Frey, Gross, and Connell,[3] and particularly upon the proposition that sawdust is necessarily a pathologic antecedent of telangiectasis. It appears from the tabulation of incidence of telangiectasis and sawdust in various localities that one cannot accept results applicable to one area as being necessarily applicable to the nation as a whole. Two factors of incidence of the conditions may influence the interpretation of results gained from a given locale; (1) absolute concentration of pathologic conditions; (2) ratio of incidence of the conditions. Thus, it may be seen that in Omaha and Chicago, the concentration of telangiectasis and sawdust is relatively high and the proportional incidence of the two conditions approaches unity. With the conditions existing in these relations, it might be deduced that the con-

ditions are closely associated if not interdependent. (Getty[2] states that sawdust is a pathologic antecedent of telangiectasis.) However, this need not be the case. Sawdust may not be the primary pathologic basis for the development of telangiectasis, since, in San Francisco, telangiectasis appears uncomplicated and apparently unrelated to sawdust. Sawdust is uncommon in the San Francisco Bay area; in fact, no lesions were seen during the period of observation and sampling. These facts indicate that, although well-developed telangiectasis may be the result of sawdust condition in the areas studied by Getty, it need not be preceded by those pathologically responsible for sawdust. Telangiectasis should, therefore, be considered as a pathologic entity which may arise from numerous causes which have as a common factor liver parenchymal destruction.

SUMMARY

1) Gross, subgross, and microscopic observations of lesions of telangiectasis, as seen in the San Francisco Bay region, are reported.

2) The results of subgross studies demonstrate that telangiectasis is limited to the hepatic portal system.

3) Vascular injections demonstrate that the lesions of telangiectasis are not restricted to sinusoids. By enumerating the branches of the hepatic portal vein, it has been demonstrated that well-formed lesions may involve intralobar branches of the portal vein as large as the third order of size.

4) Correlation of subgross and microscopic studies demonstrate that telangiectasis arises as a result of parenchymatous degeneration which may be due to varying causes. The dilation of the blood vessels is a secondary reaction.

5) A second type of telangiectasis is described in fluke-infected livers, giving further evidence that telangiectasis is but a pathologic classification and may arise from various causes.

6) From the standpoint of pathology and incidence, there is no discernible correlation between telangiectasis and sawdust in the San Francisco packing area.

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Persistence of *Brucella Abortus* Infection in Cattle

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THE PERSISTENCE of infections produced by the 3 Brucella species has not only been a debatable question but is also a very important one from the standpoint of prevention, control, and eradication. Recent studies at the Animal Disease Station at Beltsville, Md., on brucellosis in swine have shown that genital infections may persist in sows for at least thirty-six months and in boars for fifty-one months. Blood stream infection has been demonstrated for as long as thirty-four months following exposure. Similar studies have been conducted on goats naturally infected with *Brucella melitensis*. Urogenital, blood stream, and udder infections may persist in goats for at least two years. A persistent bacteriemia was also found in castrated goats. The results obtained from studies on *Brucella suis* and *Br. melitensis* infections in their natural hosts demonstrated the necessity of reviewing past work and initiating further studies on persistence of *Brucella abortus* infection in cattle.

REVIEW OF LITERATURE

Many previous investigators have demonstrated that *Br. abortus* infections persist in the udders of some cows for years. Fitch, Bishop, and Kelly,[6] in 1936, reported that a bacteriemia is not common in cattle with chronic brucellosis but does occur frequently in animals recently infected. These findings are similar to those of Lübke,[10] who reported that a bacteriemia existed in 90.4 per cent of 21 recently infected cows and in only 5.9 per cent of 81 chronic cases. Soule[11] made two series of hemoculture studies on large numbers of cattle showing agglutinins in the blood. In the first series of tests, 299 Brucella recoveries were made from the blood of 2,237 animals and in the second series of tests, 206 positive blood recoveries were made from 2,607 cattle.

Cotton,[2] in 1913, reported that 1 cow had shed abortion bacilli from the udder for four years and three months. *Bacillus abortus* was also found in the vaginal scrapings of 1 cow for thirteen days following abortion and in another cow for forty-six days following abortion. Furthermore, the infective organism may be present in the placental and uterine discharge at the time of a normal parturition subsequent to an abortion. In 1919, this same investigator[3] reported on further work conducted along similar lines and found that *B. abortus* was consistently recovered from the udder secretions of a cow for six and one-half years and from the uterus of another cow fifty-one days after abortion.

Following this work, Fitch, Delez, and Boyd[4] demonstrated that the abortion bacillus could be isolated from the vagina of cows for twenty-five days following abortions or full term parturitions. A subsequent report in 1932 by Fitch, Bishop, and Boyd[5] showed that *Bacterium abortus* was isolated from the urine of cows twelve days following abortions or full term parturitions. However, they also stated that this organism was not found frequently in the urine and feces of infected cattle. Further work on localization of *Br. abortus* in the bovine uterus was reported by Fitch, Boyd, Bishop, and Kelly,[8] in 1939. Ninety-three nongravid uteri were examined and *Br. abortus* was isolated from 24 (36%) of the 66 showing no evidence of recent parturitions and from 23 (85%) of 27 showing evidence of recent parturition. Forty-one gravid uteri were examined and *Br. abortus* was not recovered from the ones in which pregnancy of less than four and one-half months existed. However, the infective organism was isolated from 13 (45%) of those over the four and one-half month of gestation. The maximum period for which *Br. abortus* was shown to persist in a nongravid uterus following parturition was 195 days.

Birch and Gilman[1] reported finding *Br. abortus* in the genital mucosa of 4 artificially infected cattle for fifteen, thirteen, eight, and seven months, respectively, follow-

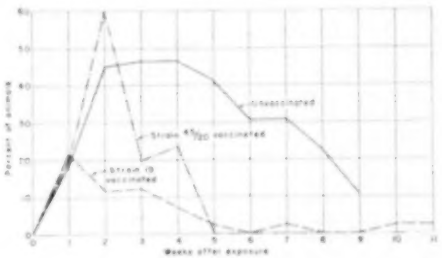

Fig. 1—Relation of bacteriemia to the degree of resistance in artificially infected cattle from time of exposure to period of mean parturition.

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ing calving. Regarding the presence of *Br. abortus* in the uterine material at the time of full term parturitions of reacting cattle, Gwatkin[5] found the infective agent in approximately 20 per cent of the cases examined, which was similar to the results obtained by Fitch *et al.*[3] Furthermore, the last mentioned authors recovered *Br. abortus* from the

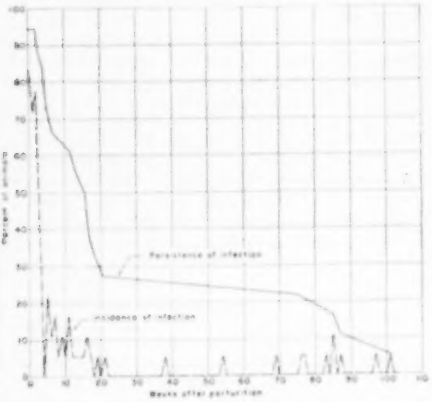

Fig. 2—Incidence and persistence of urogenital infection in 18 artificially infected cows.

vaginas only twice from 44 examinations on 58 reacting pregnant cattle, 20 of which later showed infected placentas at the time of parturition. The explanation given is that the organisms are not likely to appear in the vagina until the uterine seal is broken.

EXPERIMENTAL METHODS

Cattle free from brucellosis were artificially exposed by applying a suspension of virulent *Br. abortus* organisms on the conjunctiva. Viability determinations were made on all Brucella suspensions immediately preceding exposure. Two highly virulent strains of *Br. abortus* were used separately or in combination for exposure purposes. One strain was aërobic and the other required 10 per cent CO_2 for growth. However, the CO_2-sensitive strain was abandoned because of the extremely long incubation period of at least fourteen days required for growth on artificial mediums. Many times this resulted in contamination of the plates with mold which interfered with interpretation of results.

Procedures for collecting and culturing bovine specimens of *Br. abortus* differ with the various laboratories. In the work reported in this paper, routine blood samples were collected in 100-ml. amounts from the jugular vein of cattle in a sterile manner. Fifty milliliters were drawn in each of two flasks containing 100 ml. of citrated veal infusion broth. The final concentration of sodium citrate was 1 per cent. These bottles were then incubated for fourteen days at 37 C. in an atmosphere of 10 per cent CO_2. Following incubation, the bottles were thoroughly shaken and 0.2 ml. of the blood and broth was inoculated on the first of three serum-potato agar plates. This inoculum was serially diluted on the plates by means of a platinum wire spreader. The plates were then incubated under an atmosphere of 10 per cent CO_2 for seven days, at which time they were observed for Brucella growth. All plates showing no evidence of growth were reincubated for seven more days. Both plate- and tube-agglutination tests were conducted on the blood serum specimens. End point titrations were determined on all samples by the tube method.

Aborted fetuses, uterine material, colostrum samples from each quarter of the udder, and blood were collected as soon as possible follow-

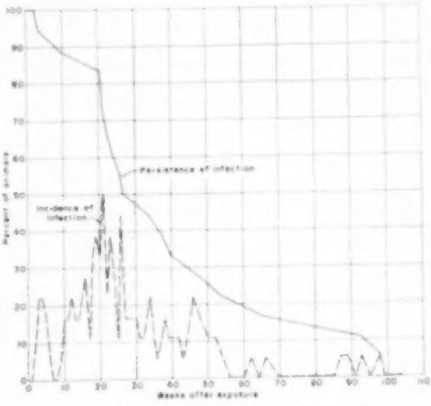

Fig. 3—Incidence and persistence of blood stream infection in 18 artificially infected cows.

ing parturition. All specimens except blood were cultured on serum-potato agar in an atmosphere of 10 per cent CO_2. A total of five organs and six portions of the digestive tract were cultured from each aborted fetus. Each of 6 guinea pigs was injected subcutaneously with 0.25 ml. of the uterine discharge. Five milliliters of colostrum from each quarter of the udder were injected intraperitoneally in each of 2 guinea pigs. Four to five weeks following inoculation, the guinea pigs were destroyed for observation of lesions, collection of blood for agglutinin determinations, and spleens for bacteriologic studies.

Vaginal specimens were routinely collected on sterile cotton swabs, in duplicate. The

external genitalia were thoroughly washed with soap and water followed by swabbing with 70 per cent alcohol solution. The lips of the vulva were then separated, and large cotton swabs, attached to a 24-in. No. 9 wire, were inserted into the vagina, for collecting the material. Each of the two swabs was washed in 0.85 per cent saline solution and cultured separately by inoculating 0.2 ml. of the washing on the first of a series of three serum-potato agar plates. The remaining portions of the washings from the two swabs were combined and injected subcutaneously in equal amounts in 4 guinea pigs.

Urine samples were collected with sterile rubber catheters following the collection of vaginal specimens. The urine was then centrifuged at 2,500 r.p.m. for ten minutes followed by decanting the clear urine. The sediment was resuspended in physiologic saline solution followed by the same cultural and animal inoculation procedures employed with vaginal specimens.

All recoveries were identified and classified as a check against possible outside contamination.

RESULTS

Three experimental approaches were employed in determining the persistence of *Br. abortus* infection in cattle.

The first part of the research consisted of studying the degree and persistence of blood stream infection in 270 cows from the date of exposure to the date of parturition. Although all cows were exposed by the conjunctival route, the exposure strains and dosages varied. Blood was routinely collected for culture at weekly intervals and the usual specimens were collected from each animal at termination of gestation. *Brucella abortus* was isolated from 172 cows which were exposed in the following manner. Strain 2308 (aërobic culture) was employed on 68 unvaccinated and 65 vaccinated cows and strain 2016 (CO_2-sensitive culture) on 9 animals in each group. A combination of both strains, in equal amounts, was used to expose 13 unvaccinated and 12 vaccinated cattle; 12 cows were infected with each of the strains and 1 animal with both strains. The remaining 98 cows which showed no evidence of brucellosis were exposed in the same manner and at the same time as the infected ones. No significant difference in the course of infection could be attached to the exposure strains employed when used in similar dosages; therefore the animals were classified according to their preëxposure status. The average period of gestation at the time of exposure was five months. In both figure 1 and table 1, the cows are divided into unvaccinated, strain 19-vaccinated, and strain 45/20*-vaccinated groups.

In figure 1, a mean parturition period was determined for each group of cows. The degrees of blood stream infection encountered varied considerably between the different groups. The results show that blood stream infection was much more persistent in the highly susceptible unvaccinated controls than in the strain 19-vaccinated cattle, which exhibited varying degrees of resistance. Also, the highest incidence of bacteriemia was at the second postexposure week in the cattle vaccinated with strain 45/20, which had a high abortion and infection rate. Both the incidence and persistence of blood stream infection are not only related to the degree of resistance but also to calving performance in infected cattle, as shown in table 1. This is emphasized by the low incidence and transient nature of the bacteriemia in the strain 19-vaccinated cattle, which had a low abortion rate as compared with the high incidence and/or persistence of bacteriemia and the high abortion rate in the other two groups. This is further emphasized by 87 normal calves born to the 91 resistant cows which had no demonstrable blood infection. The numbers listed under the headings "Pos. Hem." in table 1 represent those animals which showed a postexposure bacteriemia one or more times.

At termination of gestation, *Br. abortus* was also demonstrated in the blood in 33 unvaccinated controls, in 5 strain 19-vaccinated animals, and in none of the strain 45/20-vaccinated animals. Eighty-seven per cent of these blood recoveries occurred in animals at the time of abortion. In the 3 cows which were Brucella-free at the time of normal parturitions, 1 control cow had a bacteriemia only at the fifth postexposure week and the 2 strain 19-vaccinated cows only at the first postexposure week. The blood serum-agglutination titer in the control increased from negative to positive at 1:400 by the eighth postexposure week, then progressively decreased to negative by the time of normal calving. In the 2 strain 19-vaccinated cows, the agglutination titer in 1 increased from negative to positive at 1:50 by the sixth week following exposure and the other increased from a preëxposure titer of 1:100 to 1:800 by the eighth week. The blood serum-agglutination titers in both these animals returned to their preëxposure status by calving time.

In the second part of the research, the persistence of infection was studied for a

**Brucella abortus* strain 45/20 is the vaccine strain employed by Dr. A. D. McEwen of England. This Brucella strain is characterized by its failure to produce agglutinins in the blood of cattle.

number of pregnancies in both artificially and naturally infected cows, as shown in table 2. Both groups were maintained according to the practices on the average dairy farm. All the cows in both groups had completed two pregnancies and the majority had completed three. Routine laboratory studies were conducted on specimens from each animal at termination of gestation. Blood and milk samples were collected occasionally for agglutination titer and bacteriologic determinations. Twenty-one of the artificially infected cows were exposed with an aërobic strain, 7 with a CO_2-sensitive strain, and 9 with a combination of both strains. Sixteen of the naturally infected cows were from one herd.

The degree of blood and genital infection in both groups was highest at termination of the first gestation. These conditions were associated with a high abortion rate. However, blood stream infection was slightly more persistent and the number of cows having successive abortions was slightly higher in the naturally infected group. As was normally expected, the number of abortions and the amount of bacteriemia decreases when the disease becomes chronic.

Eight animals from each group became ceased-shedders (cease to eliminate from the body) of *Br. abortus*. In the artificially infected group, 2 cows which never showed more than a suspicious agglutination reaction remained suspects and, of the 6 reactors, 1 became a suspect and 5 remained reactors. Findings in the naturally infected group of reacting cattle were similar in that 1 became negative, 1 became a suspect, and 6 remained reactors. Except for the 16 animals which ceased to shed *Br. abortus*, the remaining cows continued to eliminate the organism from the udder during the entire course of study. No evidence of cross infection in the cattle artificially exposed with the different strains could be demonstrated. Furthermore, no reinfection was demonstrated in the ceased-shedders.

Three cows from the two groups were interesting cases for study. One cow in the naturally exposed group aborted five successive times followed by four normal parturitions. *Brucella abortus* was isolated from the uterine discharge at each abortion and also at the time of the sixth and ninth parturitions. Uterine infection can be absent for one or more calvings and reappear at the time of subsequent calvings or abortions. In the 4 cows in which this phenomenon occurred, large numbers of *Br. abortus* were eliminated from the genital tract for various periods following the recurrence of uterine infection. A second cow, which received equal amounts of two strains of *Br. abortus* (1 aërobic and 1 CO_2 sensitive) through the conjunctiva, became infected with both strains. The aërobic strain localized in the right rear quarter, and the CO_2-sensitive strain in the left rear quarter. Each strain persisted in these respective quarters through seven pregnancies. A third cow, artificially exposed, never exhibited a blood serum-agglutination titer of over 1:50 at the height of infection. Brucella was recovered from the uterine material at time of normal calving.

Our findings indicate there is little difference in the course of *Br. abortus* infection between cows artificially infected through the conjunctiva and those infected by natural contact.

The third and last part of the research was made to determine the frequency with which *Br. abortus* is isolated and persistence of blood stream, urogenital, and udder infections in cows over a period of two years. Because of the similarity observed in the course of *Br. abortus* infection in artificially and naturally exposed cows, 18

TABLE 1—Relationship of Blood Stream Infection to Calving Performance in Artificially Brucella-Infected Cows

Pre-exposure Status of Cows	No. Cows	No. with Pos. Hem.	Infected at Parturition						Not Infect. at Part.	
			Abortions		Weak Calves		No. F.T. Calves		No. F.T. Calves	
			Pos. Hem.	Neg. Hem.	Pos. Hem.	Neg. Hem.	Pos. Hem.	Neg. Hem.	Pos. Hem.	Neg. Hem.
Un-vaccinated	89	68	62	14	4	3	1	4	1	0
Strain 19 Vaccinated	67	27	18	20	5	10	2	10	2	0
Strain 45/20 Vaccinated	16	10	10	5	0	1	0	0	0	0

There were 31 strain 19-vaccinated, 3 strain 45/20-vaccinated, and 4 unvaccinated cattle which resisted the exposures employed. No evidence of blood infection was demonstrated in any of the cows and all had normal calves, except 4. Three cows had weak calves and 1 aborted.

Pos.Hem.=recoveries of *Brucella abortus* from the blood; Neg.Hem.=no recoveries of *Brucella abortus* from the blood; Infect.=infected; Part.=parturition; F.T.=full term.

infected, first calf heifers were selected from a group of animals exposed by the conjunctiva with strain 2308 (aërobic). These animals received either 370×10^3 or 740×10^3 virulent organisms, which produced infection in 77 per cent and 89 per cent of the unvaccinated controls, respectively. Bacteriologic and serologic studies were conducted on the blood for 104 weeks beginning at time of exposure and on the urogenital tract for 103 weeks beginning at time of parturition. Specimens were collected weekly for forty-six weeks and biweekly thereafter. Vaginal specimens were not collected until after parturition so as not to interfere with termination of gestation. Urine samples were collected for only six months because of the appearance of extraneous bladder infection. On only a few occasions was *Br. abortus* isolated from the urine and not from the vagina. There is a great possibility that the urine recoveries were the result of contamination from passing the catheter.

Genital infection was demonstrated in only 15 of the 18 cows at the termination of gestation, as shown in table 3. However, *Br. abortus* was isolated from the urogenital tract of 2 more of the animals subsequent to parturition. Infection was not demonstrated in the urogenital tract of the remaining cow at any time. The last 3 cows discussed were held in isolation following parturition to eliminate the possibility of added exposure. These long incubation periods appeared to be associated with relatively small exposure doses of virulent *Br. abortus* organisms.

Fifteen of the 18 cows were held over for a second parturition, 3 of which were nonbreeders. The 3 other animals were destroyed because of *Mycobacterium paratuberculosis* infection. Of 12 cows which conceived, 8 had normal calves and 4 aborted, as shown in table 3. *Brucella abortus* was isolated from the uterine material of 1 cow at the time of aborting, from another cow at the time of normal calving, and from a third cow shortly after normal calving. No specific causes could be demonstrated as being responsible for the other three abortions.

In figure 2, the curve demonstrating the persistence of urogenital infection in cows is based on the last Brucella recovery made from each animal after parturition. There is a rather rapid decrease in urogenital infection for five months, after which the decline is more gradual. The incidence of Brucella recoveries was highest shortly after parturition followed by a sharp decline at the fourth week. Recoveries, after this time, were made at less frequent intervals, indicating the transient or intermittent nature of genital infection. The increase in number of recoveries of *Br. abortus* between eighty and ninety weeks occurred shortly after termination of the second gestation.

The animal with the most persistent urogenital infection and the highest frequency of recoveries would be classified as a repeat

TABLE 2—Findings in Naturally and Artificially Brucella-Infected Cattle

Preg. No.	No. Cows	No. Abortions	No. Br. abortus Recoveries			No. F.T. Part.	No. Br. abortus Recoveries		
			Uterine Material	Colostrum	Blood		Uterine Material	Colostrum	Blood
24 Naturally Infected Cows									
1	24	19	19	16	7	5	1	5	0
2	24	9	7	8	4	15	2	11	0
3	13	5	3	5	1	8	0	6	0
4	10	1	1	1	0	9	0	6	0
5	4	1	1	1	0	3	0	1	0
6	1					1	1	1	0
7	1					1	0	1	0
8	1					1	0	1	0
9	1					1	1	1	0
38 Artificially Infected Cows									
1	38	34	34	32	14	4	4	3	2
2	38	11	8	9	2	27	5	24	1
3	29	1	0	1	0	28	2	21	0
4	9					9	0	6	0
5	6					6	0	3	0
6	3					3	0	1	0
7	2					2	0	1	0

Preg. = pregnancy; F.T.Part. = full term parturition.

or nonbreeder. Estrus was very irregular in nonbreeder animals and the cycle varied from seventeen to thirty-two days. Recoveries of Brucella from the vagina were not necessarily associated with any particular phase of the estrual cycle.

The state of pregnancy during the second gestation period had no apparent influence on the course of blood infection but appeared to alter the course of genital infection. No isolations of Brucella were obtained from the genital tracts between breeding and parturition dates, although evidence of infection was demonstrated at the time of the second parturition or shortly thereafter in 3 of the 12 cows.

Blood stream infection persisted in over 80 per cent of the cows for five months following exposure, as shown in figure 3. The greatest number of recoveries were likewise made during this same five-month period which followed termination of gestation. In 6 cows, *Br. abortus* was isolated from the blood for three to five weeks in succession, followed by the absence of infection for various periods of time. This condition recurred several times in these animals. Both the incidence of Brucella recoveries and the persistence of infection gradually decreased for the next nine months, when there was an obvious leveling off period. The curve showing the persistence of bacteriemia is based on the last Brucella recovery made from each animal after exposure.

Bacteriemia was more prevalent at termination of the first gestation than at the second. In both cases, recoveries of Brucella were usually associated with cows that aborted or had weak calves. However, one

TABLE 3—*Brucella Abortus* Recoveries and Course of the Disease in 18 Artificially Infected Heifers

	First Parturition						Second Parturition						Blood		Uro-genitals	
Cow No.	Term. of Gest.	Blood Aggl. Titer	Hem.	Ut. Mat.	Fet.	Col.	Term. of Gest.	Blood Aggl. Titer	Hem.	Ut. Mat.	Fet.	Col.	No.	Days After Expos.	No.	Days After Part.
1	A	400	-	+	+	+	NP	0	0	0	0	0	3	25	3	35
2	A	200	+	+	+	+	A	3200	-	-	-	+	26	277	6	143
3	A	3200	-	+	+	+	N	12800	-	-	-	+	1	19	4	115
4	WC	50	-	+	-	-	A	200	-	-	-	+	11	458	3	108
5	WC	400	-	+	+	+	N	200	-	-	0	+	5	151	5	29
6	WC	400	+	+	+	+	A	3200	-	-	-	-	3	67	6	127
7	A	1600	-	+	+	+	A	1600	+	+	-	+	8	648	6	595
8	WC	50	-	-	-	-	NP	0	0	0	0	0	5	236	1	112
9	N	25	+	+	0	+	N	1600	-	-	0	+	14	690	7	610
10	A	1600	-	+	+	+	NP	0	0	0	0	0	9	333	11	707
11	N	200	-	+	0	+	N	400	-	-	0	+	2	145	4	46
12	WC	400	+	+	+	+	D	0	0	0	0	0	10	151	4	32
13	A	200	+	+	+	+	D	0	0	0	0	0	24	264	7	83
14	A	800	-	+	+	+	N	400	-	+	0	+	6	180	6	523
15	WC	Neg	-	-	-	-	N	400	-	-	0	+	3	187	0	0
16	N	Neg	-	-	0	-	N	800	-	-	0	+	5	167	3	93
17	WC	Neg	-	+	+	-	D	0	0	0	0	0	5	375	4	16
18	A	3200	-	+	+	+	N	1600	-	-	0	+	1	155	4	17

Term. of Gest.=termination of gestation; Aggl.=agglutination; Hem.=hemoculture; Ut.Mat.=uterine material; Fet.=fetuses; Col.=colostrum; Expos.=exposure; Part.=parturition; A=abortion; WC=weak calf; N=normal; NP=not pregnant; D=destroyed; —=*Brucella abortus* not isolated; +=*Brucella abortus* present; 0=no specimen available; Neg.=negative.

such recovery was obtained from the blood of 1 cow at the time of normal calving. A complete history on recoveries of Brucella from the blood is shown in table 3.

In most animals, *Br. abortus* was isolated more frequently and for a longer time from the blood stream than from the urogenital tract. Bacteriemia persisted for an average of 251 days in each cow as compared with 183 days for genital infection. Maximum blood-agglutination titers varied from 1:3,200 to 1:12,800. Recoveries from the blood were not necessarily associated with recoveries from the urogenital tract or with a change in concentration of blood agglutinins.

Udder infection was demonstrated in 17 of the 18 cows at sometime during the experiment. Infection persisted in the udders of 16, whereas the other cow had apparently recovered by the time of autopsy, twenty-six months after exposure. This cow had a blood serum-agglutination titer of 1:100 at the time of autopsy. *Brucella abortus* was not isolated from the udder of the remaining cow over a period of thirteen months when the animal was destroyed because of paratuberculosis infection.

A study was made of the sites of localization of infection in 15 of the cows which have been autopsied. The average agglutination titer at this time was 1:1,200. No Brucella was recovered from 2 animals although both had blood serum-agglutination titers of 1:100 or higher. Infection in the udder was always associated with infection in the supramammary lymph glands. *Brucella abortus* was recovered from the uterus of a nonbreeder cow 101 weeks following parturition. This animal was the most persistent shedder of infective organisms from the genital tract. The 3 remaining living cows have chronic udder infection. Tissues from which recoveries were made and the percentage of recoveries are shown in table 4. No recoveries of *Br. abortus* were made from the spleens, livers, kidneys, ovaries, vaginas, bile, urine, or mesenteric and iliocecal lymph glands.

DISCUSSION

In experimental studies, the incidence and persistence of bacteriemia may be used as criteria for aiding in estimating the degrees of susceptibility or resistance of exposed cows to virulent *Br. abortus*. When the amounts and virulence of the exposure were comparable, bacteriemia was not demonstrated in the fully resistant strain 19-vaccinated cows. On the other hand, in the highly susceptible unvaccinated controls, there was a high incidence and persistence of blood stream infection. Strain 19-vaccinated animals not fully protected but with varying degrees of resistance showed a transient bacteriemia. The higher incidence of Brucella recoveries from the blood were more frequently associated with abortions and weak calves than with full term parturitions.

Blood stream infection persisted for five months in a high percentage of recently infected unvaccinated cattle. As the disease becomes chronic, the number of animals having bacteriemia decreases rapidly. However, approximately 5 to 10 per cent continue to have an intermittent blood infection for at least two years. This was demonstrated in cattle examined at the time of parturition for several pregnancies and those examined at regular intervals for two years. These findings compare favorably with those of Soule[11] and Fitch *et al.*[6]

Occasionally, an animal is encountered with a transient bacteriemia and rise in blood agglutinins with no further evidence of the disease. This phenomenon is apparently uncommon in bovine brucellosis but occurs frequently in swine brucellosis.

Furthermore, the fact that 3 of the cows showed no evidence of infection until after calving stresses the need to continue the examination of experimental animals beyond this time. No doubt animals having a long incubation period associated with the disease have been overlooked in the past.

Our failure to demonstrate a bacteriemia in some animals should not be interpreted as an absence of blood infection. Isolating *Br. abortus* from the blood of cows is dependent on the amount of blood collected, frequency of collections, and the elimination of extraneous contamination at the time of collecting. In many cases of bacteriemia, apparently, there are only a few infective organisms circulating in the blood stream. This is demonstrated by making recoveries from only one of two 50-ml. portions of blood drawn simultaneously.

It is common knowledge that cattle are most dangerous at the time of parturition

TABLE 4—Localization of *Brucella Abortus* in 15 of the 18 Artificially Infected Cows

Tissue	Percent
Udder	86.66
Uterus	13.33
Cervix	6.66
Lymph glands	
Supramammary	86.66
Iliacs	40.0
Prescapular	20.0
Prefemoral	13.33
Submaxillary	13.33
Retropharyngeal	13.33
Parotid	13.33
Bronchial	13.33
Gastrohepatic	13.33

or shortly thereafter from the standpoint of eliminating *Br. abortus* from the genital tract. However, the nonbreeder or, more correctly classified, repeat breeder appears to have been overlooked as a spreader of brucellosis. Our findings compare very favorably with those of Fitch *et al.*,[8] in that infection was demonstrated more frequently in the nongravid than in the gravid uterus. The failure to isolate Brucella from the vaginas of cows during pregnancy was probably due to the presence of a uterine seal. This idea has been suggested by other investigators as the explanation for this phenomenon.

Slightly over 80 per cent of the cows studied persistently eliminated *Br. abortus* in the vaginal discharges for one month following parturition. The incidence of recoveries and persistence of genital infection, following this period, decreased rapidly for the next four months after which time conditions remained rather constant. Recoveries at this time were infrequent and the number of organisms isolated varied considerably. Improved collecting techniques and more frequent collections would no doubt have resulted in making more Brucella recoveries.

Another debatable and often discussed question is the similarities or differences between the course of brucellosis in artificially and naturally infected cattle. Our results indicate there is very little or no difference in the character of the disease in the two groups. The abortion rate, degree of genital, udder, and blood infection, and number of ceased-shedders are so nearly alike that only minor differences can be demonstrated. In our experience, the amount and virulence of the exposure strain and the susceptibility of the individual have a greater degree of influence on the course of infection than methods of exposure.

SUMMARY

1) In cows artificially exposed with virulent *Brucella abortus*, the degree of blood stream infection was related to susceptibility and or resistance of the animals and calving performance.

a) A high incidence and persistence of bacteriemia were associated with highly susceptible animals and a high abortion rate.

b) A low incidence and persistence of bacteriemia were associated with cows which became infected but showed varying degrees of resistance. Furthermore, bacteriemia was not demonstrated in cows which had complete resistance against exposure.

c) A transient bacteriemia was encountered in 3 exposed cattle where no other evidence of infection was demonstrated. This condition is apparently rare.

2) No major differences could be demonstrated in the course of brucellosis between artificially and naturally infected cows.

3) In 18 artificially infected cattle, blood, genital, and udder infections were studied for two years.

a) The degree of Brucella infection in the blood and genital tract was highest near termination of the first gestation following exposure.

b) *Brucella abortus* was demonstrated in the blood stream of 1 cow for as long as ninety-seven weeks and in the genital tract of another cow for 101 weeks.

c) Genital infection was the most persistent in a repeat breeder cow.

d) *Brucella abortus* was not isolated from the genital tract of 12 cows between conception and second parturition. However, 3 of these animals had genital infection at the time of the second parturition or shortly thereafter.

e) Recoveries from both the blood and urogenital tract were made at irregular intervals.

f) The estrual cycle was very irregular in the cows classified as repeat breeders or nonbreeders.

g) Recoveries of Brucella from the vagina were not necessarily associated with estrus.

h) *Brucella abortus* localizes most frequently in the udder and supramammary lymph glands of cattle.

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Studies with the Johnin and Tuberculin Intradermal Tests in Cattle Naturally Infected with *Mycobacterium Paratuberculosis* (Johne's Disease)

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PART I

Studies with the Intradermal Johnin Test

JOHNE'S DISEASE is a chronic infectious disease caused by *Mycobacterium paratuberculosis*. The disease is characterized by inflammation of the intestinal tract and mesenteric lymph glands, loss of condition, and diarrhea. Cattle and sheep are the more common host animals; however, goats, horses, and deer have been shown to be susceptible.

The intestinal mucous membrane is the primary site of infection, which means that large numbers of the causative organism may be present in the feces of diseased animals and, therefore, the diseased animal is always a potential source of infection to other animals. Infection takes place in the susceptible animal by eating feed and drinking water which has been contaminated. Young animals seem to be more susceptible than mature ones.

A foremost consideration in the control of this or any other infectious disease is the elimination of primary infection sources, namely, the infected animals and the infected surroundings. It is, therefore, paramount to have an understanding of the infectious agent both as to its behavior within the animal and in the animal's environment. This, in turn, necessitates developing a method which will identify infected animals, also an accepted procedure for eliminating or quarantining infected animals and removing environmental sources of infection.

This is a coöperative project between the Department of Veterinary Science, Louisiana State University, Baton Rouge, and U. S. Department of Agriculture, Bureau of Animal Industry, Regional Animal Disease Research Laboratory, Auburn, Ala.

Dr. Sikes was formerly at the Department of Veterinary Science, Louisiana State University, where the experimental work was done. At present, he is at the Department of Animal Husbandry and Veterinary Science, University of Tennessee, Knoxville.

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EXPERIMENTAL PROCEDURE

The hair was clipped from an area 3 by 8 in. on the side of the neck of each animal. Extra fine clipper blades were used so as to cut the hair as close to the skin as possible. A new area (unused) on the side of the neck of each animal was clipped for each test. The skin thickness in the anterior and posterior regions of these areas was measured and recorded, along with that of each caudal fold. Johnin (0.2 cc.) was injected intradermally into the right caudal fold and the posterior portion of the clipped area on the neck. Mammalian tuberculin (0.2 cc.) was injected intradermally into the left caudal fold and the anterior portion of the clipped area. All injections were made with 27-gauge, ⅝-in. needles and were at least 4 in. apart in the clipped areas. These tests were made simultaneously, and at three-month intervals. The caudal fold was used continuously, but the injections on the neck were made in a new site at each test.

The johnin used in these tests was made by the U. S. Department of Agriculture, Animal Disease Research Laboratory, Auburn, Ala. It was a "pooled" johnin made from synthetic medium on which 13 strains of *M. paratuberculosis* had grown for twelve weeks. The method employed to prepare this johnin was similar to that used by Dorset and Henley[1] to prepare tuberculin from cultures of *Mycobacterium tuberculosis*. After preparation from the various strains, the johnins were mixed thoroughly and designated "pooled XYZ." The tuberculin used in these tests was made by the Pathological Division, Bureau of Animal Industry, U. S. Department of Agriculture.

The Kummer and Johnson[2] dermal thickness gauge was used for all skin measurements. Millimeter readings were made of the skin thickness of each area before injection and of the injection sites at forty-eight and seventy-two hours after injection. All increases of 3 mm. or more in skin thickness at injection sites were classed as positive and designated by the plus sign (+). All increases from 2 to 3 mm. were classed as suspicious and designated by the plus and minus sign (±). An increase of less than 2 mm. was classed as negative and designated by the minus sign (−). This classification closely approaches

field use on routine tuberculin testing and is used by other investigators.

HERD HISTORY

The herd was composed of about 400 animals, approximately one half of which were calves and yearlings, representing Jerseys and Holstein-Friesians in about equal numbers. The majority of the herd sires and a few Jersey cows originated from herds out of the state. In all cases, these newly acquired animals were negative to the intradermal tuberculin test when added to the herd, and came from herds which were tuberculosis-free accredited.

The pasture soil is alluvial, black, and typical of the Mississippi river bottom, which is very high in organic matter. The topography is flat and consequently drainage is poor. Water stands in the shallow, flat bottom ditches in the pastures nearly one half the time.

Johne's disease has been known to exist in this herd since 1927. The average yearly mortality from this disease has ranged from 1 to 5 per cent, being confined in most instances to adult cows 4 years of age or older. There has not been a significant breed difference in the incidence of clinical cases or mortality.

In 1940, the two herds (No. 1 and 2) were tested with johnin injected intradermally into the caudal fold. All cattle reacting to this test were placed in herd 1, and all cattle negative to the test were placed in herd 2. The barn, sheds, stalls, and equipment in which herd 2 was maintained were thoroughly scrubbed with hot lye water, rinsed, and washed down with 3 per cent solution of compound cresol. This practice was not continued during the war years due to labor shortage. All manure was removed from the holding pens and general sanitary precautions were observed.

All newborn calves were allowed to remain with their dams in clean box stalls for twenty-four hours after parturition. Then, the calves were removed to a calf barn, hand-fed from individual pails, permitted the use of a lot for exercise, and maintained as a separate unit until weaning time. They were then moved to a pasture which formerly had been used by the adult cattle. This pasture was plowed with a tractor-drawn turning plow before any calves were permitted to enter it. The heifers stayed in this pasture until they were about 1 year old, and were then moved to a second pasture which also had been used by adult cattle. This pasture also was plowed similarly to the preceding one before the first yearling heifers were placed on it. They were kept here until time for breeding, when they were moved to a third pasture which had been treated similarly to the above mentioned ones. All heifers remained in this pasture until a few hours prior to the termination of pregnancy. Two weeks after parturition they were added to the milking herd.

All cattle which reacted to the test were placed in herd 1 because the barn, pastures, holding lots, and equipment had been in use many years, whereas, those of herd 2 were relatively new. It was thought that the contamination in and around the barns and pastures with the Johne's bacillus was much greater in herd 1 (long-used premises).

Two tests were made each succeeding year on both the reactor and negative herds. Many of the animals in herd 1 (reactor herd) failed to react on subsequent caudal fold tests, or reacted irregularly, while a few new reacting animals occurred on each test of herd 2 (negative herd). All new reacting animals were placed in herd 1 immediately after the positive test readings were made. The caudal fold was used continuously for the testing site and all test readings were made forty-eight hours after injections. In January, 1945, the number of cattle in herd 1 had become so great, due to the addition of new reactors from herd 2, and to the normal increase from its own reproduction, that accommodations were exhausted and no more additions could be made. As a result, all subsequent reactor cattle occurring in herd 2 have been maintained there.

This herd of cattle was first accredited for tuberculosis in 1925 and has been free from the disease since that date. No pathologic lesions suggestive of this disease have ever been found at autopsy of any animal from this herd.

EXPERIMENTAL RESULTS

All of the cattle included in the 430 tests had given positive reactions on the caudal fold when they were added to this herd. It is interesting to note that only 37 of all 430 tests completed were positive on the caudal fold at forty-eight hours, and 19 at seventy-two hours. However, there were 325 positive tests out of 430 completed on the neck at forty-eight hours, and 212 positive at seventy-two hours. There were 282 negative tests on the caudal fold at forty-eight hours, and 352 at seventy-two hours, as compared to 43 negative tests on the neck at forty-eight hours and 91 at seventy-two hours. These 43 negative tests on the neck at forty-eight hours after injections were made were all suspicious or positive at seventy-two hours.

There were 45 positive tests on the caudal fold at forty-eight hours, and 29 at seventy-two hours after injection, as compared to 338 at forty-eight hours and 238 at seventy-two hours on the neck. Of the 425 tests completed, 256 were negative at forty-eight

hours and 323 were negative at seventy-two hours on the caudal fold. However, in contrast, there were only 12 negative on the neck at forty-eight hours and 69 at seventy-two hours. These 12 negative neck tests at forty-eight hours became suspicious or positive at seventy-two hours.

Many animals in herd 1, all of which had reacted at some time, gave very irregular reactions to the johnin intradermal tests on the caudal fold when made at six-month intervals from 1940 to 1944. Hagan[3] has suggested the possibility of reinfection from the pasture, which is highly probable

TABLE 1—Johnin Test Results in Herd 1

No. of tests	Reactions on caudal fold (48 hr.)			Reactions on caudal fold (72 hr.)			Reactions on posterior neck (48 hr.)			Reactions on posterior neck (72 hr.)		
	—	+	+	—	+	+	—	+	+	—	+	+
430												
percentage responses	282 65.6	111 25.8	37 8.6	352 81.9	59 13.7	19 4.4	43 10.0	62 14.4	325 75.6	91 21.2	127 29.5	212 49.3

There were 538 negative caudal fold tests at forty-eight hours and 675 at seventy-two hours out of 855 tests completed in these two herds. In contrast to these results, there were only 55 negative neck tests at forty-eight hours and 160 at seventy-two

under these herd conditions and which may partially explain some of this irregularity in allergic response. On this assumption, it is possible that the more recently or actively infected animal would have a higher degree of sensitivity and that this sensitivity would

TABLE 2—Johnin Test Results in Herd 2

No. of tests	Reactions on caudal fold (48 hr.)			Reactions on caudal fold (72 hr.)			Reactions on posterior neck (48 hr.)			Reactions on posterior neck (72 hr.)		
	—	+	+	—	+	+	—	+	+	—	+	+
425												
percentage responses	256 60.2	124 29.2	45 10.6	323 76.0	73 17.2	29 6.8	12 2.7	75 17.7	338 79.6	69 16.3	118 27.7	238 56.0

hours. There were 82 positive tests on the caudal fold at forty-eight hours, and 48 at seventy-two hours. However, there were 663 positive tests on the neck at forty-eight hours and 450 at seventy-two hours.

DISCUSSION

Because there is not an entirely specific and completely satisfactory known diagnostic test for Johne's disease in the individual animal, an attempt was made to compare a continuously used testing area (caudal fold) and a new or unused area on the side of

diminish as apparent recovery from the exposure takes place. The very mild winters, excessive amounts of water, high organic content of the black, fertile soil, and dense vegetative growth of pasture plants, all go to make up a highly suitable environment for the protection, survival, and possible reproduction of the Johne's bacillus on the pastures in southern Louisiana. These environmental factors possibly play a much greater part in the course of the development of the disease in the individual animal and in the herd than most people believe.

TABLE 3—Johnin Test Results in Herds 1 and 2 Combined

No. of tests	Reactions on caudal fold (48 hr.)			Reactions on caudal fold (72 hr.)			Reactions on posterior neck (48 hr.)			Reactions on posterior neck (72 hr.)		
	—	+	+	—	+	+	—	+	+	—	+	+
855												
percentage responses	538 62.9	235 27.6	82 9.5	675 78.9	132 15.5	48 5.6	55 6.4	137 16.0	663 77.6	160 18.7	245 28.7	450 52.6

the neck in testing naturally infected cattle. In testing known Johne's disease-infected cattle with intradermic johnin, it is a common observation that they do not react on repeated tests with a uniform regularity nor do they show the same degree of reaction when compared to previous tests.

Rider[4] found that in 1,100 double intradermal tuberculin tests on the side of the neck of naturally sensitized tuberculous cattle, 90 per cent of the negative reactions had a swelling not over 2 mm. at forty-eight hours, and the total increase was not over 3 mm. In 90 per cent of the positive

reactions, the increase was more than 3 mm. at forty-eight hours and the total increase was more than 5 mm.

Due to the chronic nature and very slow progress in active pathology in Johne's disease in most animals, it is very probable that the sensitivity will never be as great, except in the advanced clinical case, as in bovine tuberculosis, which usually manifests a more active type of pathology.

Wilson, Schwabacher, and Maier[5] were able to partially desensitize guinea pigs artificially sensitized with heat-killed tubercle bacilli by daily injection of old tuberculin (OT). They believe that "desensitization probably is never complete in healthy animals." Apparently, it acts not by abolishing allergy, but by converting a "high" into a "low" degree of allergy. Possibly, the animals in herd 1, which had been tested twice each year on the caudal fold for four years, had a partial local desensitization in that area. It is evident from table 1 that the caudal fold of these animals, with only 8.6 per cent of all tests positive at forty-eight hours after injections, does not show the same degree of sensitivity as the unused area on the neck, with 75.6 per cent of all tests positive (3-mm. or more increase).

In both herds, it is evident from tables 1 and 2 that many more positive reactions occurred at forty-eight hours than at seventy-two hours after injections, when comparing the caudal fold tests to each other and the neck tests to each other. The relative percentages of each are: caudal fold positive tests in herd 1 at forty-eight hours, 8.6, and at seventy-two hours, 4.4. In herd 2, the caudal fold positive tests at forty-eight hours were 10.6 per cent and at seventy-two hours, 6.8 per cent. The positive tests in herd 1 on the neck at forty-eight hours was 75.6 per cent and at seventy-two hours, 49.3 per cent. In herd 2, a similar picture was observed. There were 79.5 per cent of all tests positive at forty-eight hours, and 56.0 per cent positive at seventy-two hours. Thus, it seems that more positive reactions occur at forty-eight hours than at seventy-two hours after injections are made. There does not seem to be a great difference in the relative percentage of positive, suspicious, or negative reactions in the two herds when comparing the caudal fold tests to each other at forty-eight and at seventy-two hours. The same is evident when comparing the relative percentage of positive, suspicious, and negative tests on the neck region of the two herds to each other at forty-eight and seventy-two hours, respectively.

As shown in table 3, the number of suspicious reactions on the caudal fold at forty-eight hours is significantly greater than at seventy-two hours, being 27.6 and 15.5 per cent, respectively. This seems to be important. An opposite trend is obvious when comparing the neck tests. For instance, there were 137 and 245 suspicious tests (16 and 28.7%), respectively.

This difference may be explained on the basis of the relative amount of sensitivity in the caudal fold (continuously used) when compared to the neck (new or unused area). It seems that, with the limited sensitivity in the caudal fold, the reactions reached their maximum in size in the great majority of cases at forty-eight hours after injections and began to subside much more quickly than those on the neck region with a greater amount of sensitivity. In most instances, the greater the sensitivity the longer the reactions persist after injections are made. A recognition of this possibility may be all important in trying to eradicate this disease when testing with johnin intradermally. This would, indeed, be a point of consideration when repeated intradermal tests are made in the same site on the caudal fold at intervals of ninety days or less.

In both herds, it is evident from tables 1 and 2 that many more positive reactions occurred at forty-eight than at seventy-two hours after injections. The optimum time to make test observations seems to be at forty-eight hours after injections are made, preferably using a new site each time. Of the 55 negative tests on the neck at forty-eight hours (table 3), 23 of them became suspicious or positive at seventy-two hours. Thus, there were only 32 of the 55 which were actually negative at both readings, and one half of this number reacted on subsequent tests. These negative neck tests occurred on animals which gave suspicious caudal fold reactions at either forty-eight or seventy-two hours. While there is no known explanation for this deviation, two possibilities present themselves for consideration: (1) It is possible that the needle point could have pierced a small blood vessel and the johnin was deposited into the general circulatory system; (2) animals sometimes move their heads and bodies violently, causing the injection to be given subcutaneously. In either case, a negative test would be expected. In contrast, 538 of the 855 tests (62.9%) were negative at forty-eight hours on the caudal fold.

Of the 855 tests, 450 (52.6%) were positive at seventy-two hours on the neck, while only 48 (5.6%) were positive on the caudal fold. Of these 48 positive reactions, 14 occurred on active clinical cases of Johne's disease, which were invariably more sensi-

tive than the nonclinical cases. The remainder occurred in young animals which were very sensitive, but showed a great diminution in sensitivity on subsequent tests.

SUMMARY

A total of 855 johnin intradermal tests were made on cattle naturally infected with Johne's disease in two herds. The tests on the caudal fold are compared to those on the neck region and support the following conclusions:

1) A greater sensitivity exists on the neck region (unused area).

2) The optimum time for making test observations appears to be forty-eight hours after injections are made.

3) A new injection site seems preferable when repeated tests are to be made at intervals of three months or less.

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PART II

Studies with the Intradermal Tuberculin Test

EXPERIMENTAL PROCEDURE

The experimental procedure is described in Part I of this report.

EXPERIMENTAL RESULTS

Table 1 gives the total number of tuberculin tests made on herd 1 and the number of positive, suspicious, and negative reactions on the caudal fold and the anterior portion of the neck area at forty-eight and seventy-two hours after injections were made. There was one positive at forty-eight hours and five at seventy-two hours on the caudal fold. In contrast, there were 73 and 25 positive, respectively, on the anterior portion of the neck.

Table 2 gives the total number of tuberculin tests made on herd 2 and the number of positive, suspicious, and negative reactions on the caudal fold and on the anterior portion of the neck area at forty-eight and seventy-two hours after injections were made. There were eight positive at forty-eight hours and ten at seventy-two hours on the caudal fold. In contrast, there were 104 and 76 positive, respectively, on the neck. There were 133 suspicious at forty-eight hours and 89 at seventy-two hours.

The total number of positive, suspicious, and negative tests on the two herds are given in table 3. There were nine positive at forty-eight hours and 15 at seventy-two hours on the caudal fold. In contrast, there were 177 and 101 positive on the anterior portion of the neck areas. The number of positive, suspicious, and negative reactions at forty-eight hours and seventy-two hours in the two testing sites (caudal fold and neck area), compare favorably in the two herds, as shown in tables 1 and 2.

DISCUSSION

It is known that a very close relationship exists between the various members of the acid-fast group of bacteria. This close relationship is manifested in their staining characteristics, morphologic features, biologic activity, and immunogenic behavior *in vivo*. From field experience in testing cattle with intradermic tuberculin, it has been observed that reactions occur which are deviations from the usually expected allergic manifestation. However, there is an increasing number of seemingly true reactions in cattle which, at autopsy, never show any microscopic pathology suggestive of tuberculosis. With these facts at hand, the experiment was outlined to try to get information on the following questions:

1) Are animals infected with Johne's disease sensitive to mammalian tuberculin, and how long does this sensitivity last?

2) Does the positive reaction persist for

TABLE 1—Tuberculin Test Results in Herd 1

No. of tests	Reactions on caudal fold (48 hr.)			Reactions on caudal fold (72 hr.)			Reactions on anterior neck (48 hr.)			Reactions on anterior neck (72 hr.)		
430	—	±	+	—	±	+	—	+	+	—	+	+
percentage responses	412 95.8	17 4.0	1 0.2	414 96.2	11 2.6	5 1.2	248 57.7	109 25.3	73 17	323 75.1	82 19.1	25 5.8

forty-eight and seventy-two hours after injection?

From 1920 to 1930, one seventh of all cattle reacting positively to tuberculin in the United States were no visible lesion (NVL) cases, according to Hastings, Beach, and Thompson.[1] It is highly probable that a larger percentage will show up in the very near future, due to the reduction in the number of advanced cases of tuberculosis and the possible widespread distribution of Johne's disease.

TABLE 2—Tuberculin Test Results in Herd 2

No. of tests	Reactions on caudal fold (48 hr.)			Reactions on caudal fold (72 hr.)			Reactions on anterior neck (48 hr.)			Reactions on anterior neck (72 hr.)		
425	—	±	+	—	±	+	—	±	+	—	±	+
percentage responses	370 87.1	47 11.1	8 1.8	395 92.9	20 4.7	10 2.4	188 44.2	133 31.3	104 24.5	260 61.2	89 20.9	76 17.9

Hastings, Beach, and Weber[2] reported that in Wisconsin, on a test of 30,010 cattle with 1.2 per cent reactions to tuberculin, there were 22.5 per cent NVL cases. In another instance, they report that 1,063 condemned cows, from herds with only 1 tuberculin reactor in each herd, gave 44 per cent NVL cases. Bruner[3] has shown that as the percentage of animals reacting to the tuberculin test decreases, the percentage of NVL cases increases.[3] Johnson, Cox, and Milligan,[4] in testing three herds consisting of 347 cattle with johnin and tuberculin, found 24 animals which reacted to johnin. Nine of the 24 johnin reactors also reacted to the tuberculin test, while none of the 313 johnin-negative cattle reacted to tuberculin. No lesions of tuberculosis were found on autopsy in any of the 9 animals reacting to both tests. All 9 had lesions considered typical of Johne's disease, and acid-fast organisms indistinguishable from *Mycobacterium paratuberculosis* were found in stained smears from intestinal mucosa of 8 animals of this group.

Studies by Coghill and Bird[5] showed marked differences in the properties of the protein of *Mycobacterium phlei* and the human strain of tubercle bacillus (H-37). For example, with protein of *M. phlei*, no precipitate occurred even in concentrated solution with acetic acid, and only about one-third as much nitrogen appeared in the aqueous extract, or in the 0.5 per cent sodium hydroxide extract, as with protein of the human tubercle bacillus. However, 14 times as much of the water-soluble nitrogen in *M. phlei* was protein nitrogen as in the case of tubercle bacillus. Thus, differences were demonstrated in the proteins of these two bacilli as well as in their nucleic acids. The nucleic acid of the human type tubercle bacillus contained thiamine, whereas that of *M. phlei* contained uracil and methylcystosine. Serologically, by means of the agglutination test, Koch[6] has shown that there is a relationship between the human tubercle bacillus and a large number of other acid-fast organisms, including some from cold-blooded animals, and that this relationship did not exist with other pathogenic bacteria. Recent work has shown that there is probably a serologic group specificity due to the polysaccharides of acid-fast bacilli, whereas, type specificity is apparently due to the protein in these bacilli. McCarter, Kanne, and Hastings[7] used the precipitin test to distinguish between proteins of the human, bovine, avian, and two saprophytic acid-fast bacteria isolated from the cow and hog.

TABLE 3—Tuberculin Test Results in Herd 1 and Herd 2 Combined

No. of tests	Reactions on caudal fold (48 hr.)			Reactions on caudal fold (72 hr.)			Reactions on anterior neck (48 hr.)			Reactions on anterior neck (72 hr.)		
855	—	±	+	—	±	+	—	±	+	—	±	+
percentage responses	742 91.5	64 7.5	9 1	809 94.6	31 3.6	15 1.8	436 51	242 28.3	177 20.7	583 68.2	171 20	101 11.8

Protein from Johne's bacillus was also distinguishable from all of these, with the exception of the avian type. Menzel and Heidelberger[8] isolated protein fractions from the living cells of *M. phlei* and the avian and bovine tubercle bacilli by the same method they had used on the human type. By means of the quantitative precipitin reaction, the corresponding fractions were distinguishable from each other, but the human and bovine types were closely related.

It is possible that the biologic potency

may be inherent in some small, relatively stable part of the protein molecule, and many changes can be effected in the molecule as a whole before there is a recognizable loss in biologic activity. It is shown that a very close relationship undoubtedly exists antigenically, by chemical fractionation of proteins, and serologically of the various members of the acid-fast group of organisms.

Out of 430 tests, there was one positive reaction to tuberculin at forty-eight hours, and five at seventy-two hours on the caudal fold in herd 1 (table 1). However, there were 17 and 11 suspicious reactions at these respective readings. Due to the size of these suspicious reactions (2- to 3-mm. increase), it is very probable that these animals would have been classed as reactors under the ordinary routine field testing for tuberculosis. Even with the limited sensitivity that existed in the caudal fold (continuously used), these numbers seem important since, in many problem herds in which a re-test is necessary before accreditation can be established, there are often borderline reactions which the veterinarian must consider as either positive or negative. However, when observing the neck reactions, a seemingly more significant and serious manifestation is evident. There were 73 positive at forty-eight hours and 25 at seventy-two hours. There were also 109 and 82, respectively, suspicious reactions. It is evident that a greater sensitivity existed on the neck area (unused) than on the caudal fold (continuously used).

A somewhat comparable observation is evident in herd 2 (table 2). There were eight positive at forty-eight hours and ten at seventy-two hours on the caudal fold, out of 425 tests completed. There were 47 and 20 reactions, respectively, suspicious. In contrast, there were 104 positive at forty-eight hours and 76 at seventy-two hours among those injected on the neck area.

From table 3 (combined test results of tables 1 and 2), it is interesting to note that 782 (91.5%) of the 855 tests completed on the caudal fold were negative at forty-eight hours. There were 809 (94.6%) negative at seventy-two hours on the caudal fold. In contrast, there were 436 (51%) and 583 (68.2%) negative on the neck area, respectively. The figures are highly significant in that 49 per cent of the cattle were either positive or suspicious on the neck tests at forty-eight hours.

At seventy-two hours, 31.8 per cent were positive or suspicious. From these data, it would seem that many NVL cases may possibly be cattle sensitized by the Johne's disease bacillus.

SUMMARY

A total of 855 mammalian tuberculin intradermal tests were made on cattle naturally infected with Johne's disease. A comparison of the tests made on the caudal fold (continuously used area) and those on the neck region (unused area) are given and support the following conclusions:

1) That animals infected with Johne's disease are sensitive to mammalian tuberculin.

2) The sensitivity seems to persist in many animals for many months, and positive reactions in many instances remain seventy-two hours or longer after injections are made.

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Pathogenesis of Bovine Mastitis. II. The Pathologic Alterations in Twenty-Five Mammary Glands

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BOVINE MASTITIS, an important disease of the dairy cow, is considered to be due primarily to infectious agents. The disease has been studied extensively from etiologic and epizoötiologic standpoints. Diagnosis, therapy, and control methods for mastitis have also been justly emphasized. The actual pathologic processes that occur in the inflamed mammary gland have received less attention.

As its primary purpose, this study was to explain the fundamental processes in bovine mastitis, especially those resulting in tissue alterations. The relationship of the lesions to clinical information was analyzed.

As employed here, mastitis refers to inflammation of the secreting alveoli and/or milk duct system of the mammary gland. Clinical mastitis is applied to those inflammations of such severity that they can be detected by ordinary methods, such as palpation, strip cup, or bromthymol blue test. The term subclinical mastitis is reserved for those inflammations of the udder not detectable by usual clinical means, but, in which, evidence of disease can be detected by more sensitive examinations, for example, cell counts. Normal milk is used to designate the secretion of the lactating gland which contains no flakes or solid material, has a pH of 6.5 to 6.8, a cell count of less than 1 million per milliliter, and a chloride content of less than 0.14 per cent. Milk that varies from such criteria is considered as abnormal and the secretion of such abnormal milk is indicative of mastitis. Acute mastitis is a clinical type of the disease with a sudden onset and a short course, whereas chronic mastitis has a protracted course.

REVIEW OF THE LITERATURE

Morrill[1, 2] reported an histopathologic study of the mammary glands of 20 cows. He described streptococcic mastitis as a chronic inflammatory process associated with areas of acute exudative inflammation. The involved areas were patchy in the upper parts of the udder and the more advanced fibrosis occurred in the lower portion. Foci of polymorphonuclear exudation were accompanied by vacuolization of the epithelium. Runnells[3, 4] found that chronic diffuse inflammation was the most common type of mastitis in cows which he examined. Proliferative changes appeared first in the ducts and later extended to the parenchyma.

Scholl and Torrey[5] made an extensive histopathologic and bacteriologic study of 92 cases of mastitis. The general types of the disease described by them were interstitial, exudative, suppurative, and fibrous mastitis. The interstitial type was most common, being found in 83 of 115 quarters which were positive for streptococci. Exudative mastitis was also commonly observed in streptococcic infections. Scholl and Torrey found considerable variation in the severity and type of inflammation in different cases and even in different parts of the same quarter. Inflammatory changes were observed in 124 of 165 quarters which were negative on culture. Gonzales[6] reported that dilatation of the alveoli, and papillary epithelial projections into ducts were commonly present in bovine mastitis. Bull *et al.*,[7] described nodular verrucose lesions of the teat canals in 39 of 203 quarters. On microscopic examination, he observed hyperplasia of epithelium and small calcareous accumulations.

Hucker[8] found pus in 51 (9%) of 560 quarters examined in a postmortem study. He observed fibrosis or induration in one or more quarters of 90 per cent of the cows. According to Nieberle and Cohrs,[9] the early gross lesions of chronic streptococcic mastitis in the region of the cistern are reddish clots on the surface of the epithelium. Later lesions included polypoid growths on the surface of the ducts and an increased amount of connective tissue. Runnells and Huddleson[4] observed that the lesions of *Bacterium abortus* infection in the udder were small foci of acute or chronic inflammation. They suggested that the changes involve the parenchyma initially and extend to the interstitial tissue.

Turner[10] mentioned that, in old cows, the superficial layer of the epithelium of the cisterns and ducts tended to become flattened and he also quoted Kaeppli, that areas of multi-

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layered squamous epithelium in the cisterns were thought to be due to the trauma of milking.

Stradtmann[11] observed severe lesions in the ventral portion of the affected mammary glands, whereas higher in the gland the lesions were less marked. In a recent report, Filion[12] described a terminal loss of function as a result of compression and atrophy of the alveoli.

Materials and Methods

Source of Cases.—The 25 cases of mastitis used for a postmortem study of the gross and microscopic tissue changes were mostly of the chronic type. Ten were from one herd previously described by Hastings *et al.*[13] These cases had been observed closely and regularly tested during the three years preceding slaughter. During the first two lactations, no streptococci were recovered from any cows in the herd by direct dilution plating on milk agar. During the third lactation and about eight months before slaughter, a cow infected with *Streptococcus agalactiæ* was introduced into the herd in the early part of the milking order. A streptococcic infection was discovered in an adjacent cow within a month and thereafter the infection spread to many animals in the herd. The approximate date of infection in each animal was determined by repeated tests made at four- to six-week intervals.

Some of the cows in this herd were reported by Hastings *et al.*[13] to be affected by the so-called nonspecific mastitis. This syndrome is described by the above authors as a clinically detectable, recurrent inflammation with moderate to marked changes in the milk and the absence of known pathogens on culture. However, staphylococci and *Aerobacter aerogenes* were found occasionally and may have been pathogenic.

In addition to cows from the herd described above, cases were obtained from the dairy herd of the University of Wisconsin. Repeated clinical examinations and laboratory tests on the milk were made on these cows at intervals before slaughter. No antemortem data were available for seven other udders obtained from a local abattoir. However, samples of milk were collected from each quarter immediately after slaughter for both bacteriologic and chemical examinations.

Clinical Methods of Diagnosis.—Examinations made in antemortem studies included both palpation of the udder and tests of the milk. The milk was tested by means of the strip cup, colorimetrically for pH, with the Hotis test, and by cultural examination. The latter usually involved streak plating of the fresh or incubated sample on either veal infusion-peptone blood agar or Difco blood agar. In some cases, a quantitative cultural examination of the freshly collected milk sample was made by smearing 0.1-ml. amounts of serial dilutions of the sample over the surface of blood-agar plates.

Representative colonies were picked from the plate cultures for isolation and further identification. The biochemical criteria used were those of Sherman[14] and Little[15] for the identification of streptococci and those of Plastridge[16] for the staphylococci. The precipitin test of Lancefield[17] was used to identify most of the streptococci.

Chemical methods were used to determine the degree of abnormality in udder secretions. The chloride and catalase titrations were made according to methods described by Johns and Hastings.[18]

Gross Postmortem Examination.—The mammary glands were collected from cows at a local abattoir within a few minutes after slaughter. Cow 20 was the single exception to this procedure; she died during a period of acute mastitis and her udder was collected about four hours after death. Within two hours after collection, the udders were taken to the laboratory. Examination was made immediately or twenty-four hours after injection of a fixing solution into the milk ducts and circulatory system. Udders from 3 cows were fixed by injection with 10 per cent formalin, while two were fixed with a saturated solution of mercuric chloride containing 10 per cent formalin. Mossman's fixative with the infusion method was used for 2 cases. The other cases were examined in the fresh state and parts of tissue fixed in Zenker's solution.

Sections were stained routinely with Harris' hematoxylin and counterstained with alcoholic eosin Y. Other sections were stained with methylene blue and basic fuchsin, Giemsa's stain, Crossman's stain, and pyronine-methyl green.

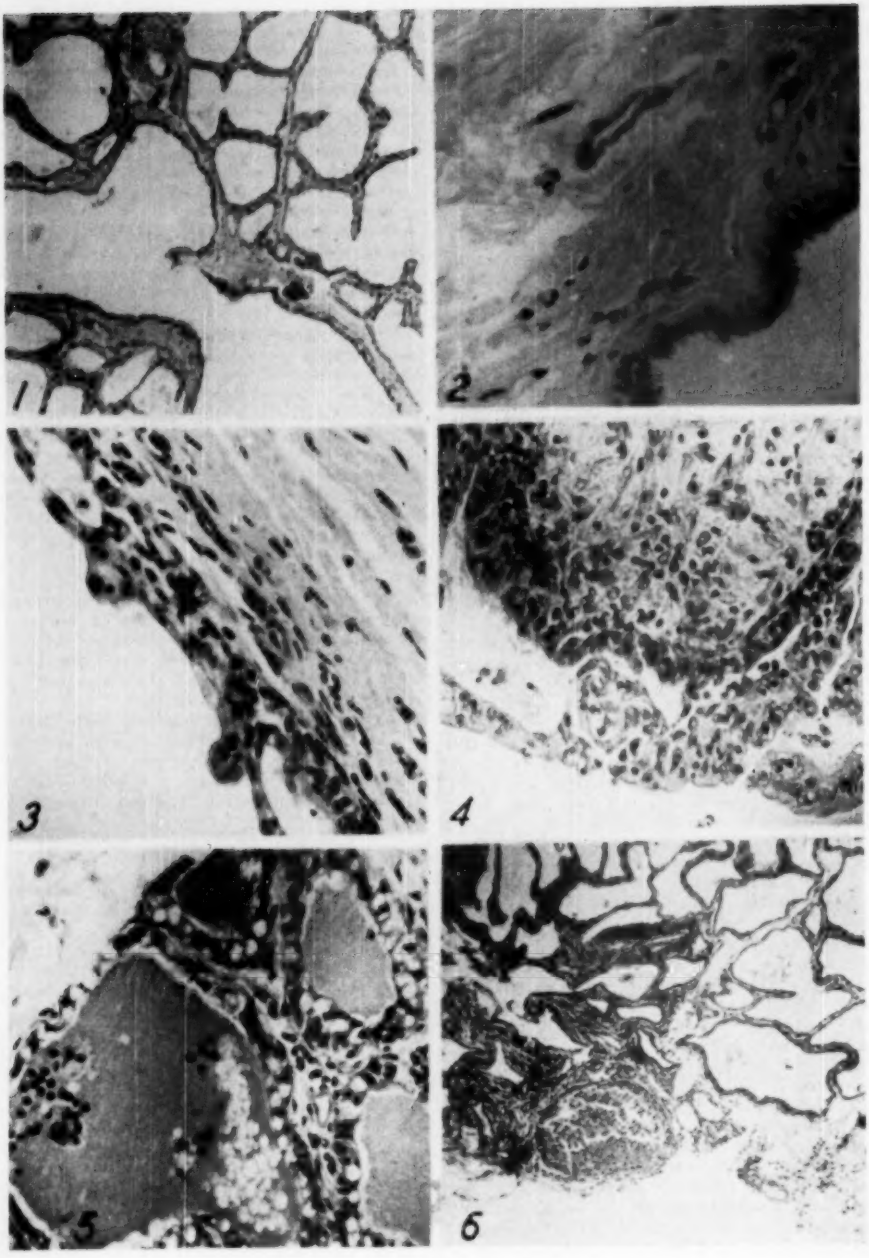
Results

The important lesions in a single quarter from a few representative animals will be described in detail.

Cow 6.—This aged, lactating Holstein-Friesian cow had been observed through three lactations and had calved for the fourth time six weeks prior to slaughter.

Right Front Quarter: Antemortem Studies.—This quarter was apparently normal until four months before slaughter. At that time, bacteriologic culture disclosed streptococci. About four weeks prior to slaughter, the quarter became swollen and produced markedly abnormal milk continuously until slaughter.

Gross Appearance.—The gland was larger than normal and of firm consistency. The cut surface was uniformly reddish brown throughout.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Microscopic Appearance.—The blood vessels were dilated. Many polymorphonuclear leucocytes were seen in both the interalveolar spaces and milk spaces. Lymphocytes and macrophages were seen in the interstitial tissue. Some lobules were slightly atrophied. Slight fibrosis was also observed in a few areas but the severe subacute inflammatory process was the predominant lesion. The epithelium of the larger collecting ducts was hyperplastic and its surface was convoluted.

Comment.—This was an example of severe streptococcic mastitis of relatively short duration. The active inflammatory process was the most prominent lesion.

Cow 20.—This 2-year-old Holstein-Friesian heifer freshened two months prior to her death and began to produce normal milk. One month after calving, a severe contusion was discovered on the distal end of the left rear teat. A deep, grayish ulcer developed, which did not improve under local treatment. About two weeks later, the left front quarter became enlarged, firm, and tender. Instead of a secretion, the product of this udder was a watery, brown, putrid material in which *Micrococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* were found on bacteriologic examination. Anorexia, elevated temperature, rapid, firm pulse, cessation of rumen movements, and other signs of severe toxemia developed within a few days. Sulfonamides, penicillin, and supportive therapy were ineffective. A gross examination was made about four hours postmortem.

Gross Appearance.—The left half of the udder was enlarged and very firm. Subcutaneous edema extended anteriorly along the abdominal wall. The left rear quarter was mottled red-gray, with a dark purple area about 5 in. in diameter around the lactiferous sinus. Many branches of the mammary vein contained thrombi, but the arteries appeared patent.

Microscopic Appearance.—Necrosis was observed in many areas. Polymorphonuclear leucocytes were numerous throughout and some macrophages, lymphocytes, and plasma cells were also present. In some areas, the epithelium was necrotic but the stroma was still intact. In other areas, the epithelium of the alveoli was partially desquamated and extensively vacuolated. Many colonies of gram-positive cocci were observed in the alveolar lumens and a few were seen in the interstitial tissue. Laminated thrombi occurred in two large veins. This was associated with dilatation of lymphatics. Extensive interstitial edema and active congestion were observed in all areas where necrosis had not occurred. Most of the milk ducts were occluded by masses of fibrin, leucocytes, and organisms.

Comment.—This case is an example of acute gangrenous mastitis following a teat injury.

Cow 22.—This lactating Holstein-Friesian heifer was observed for the first time at slaughter.

Right Front Quarter: Antemortem Studies.—A single examination of the secretion disclosed normal milk containing 420 streptococci per milliliter. No abnormalities were detected on palpation.

Gross Examination.—The quarter appeared normal in size. It had a spongy consistency and a pink color. A few scattered lobules appeared gray.

Microscopic Examination.—Two lobules contained small foci of infiltration of macrophages and lymphocytes. A few polymorphonuclear leucocytes were found in the lumens of the alveoli in this area. Six sections from other areas of the quarter contained no lesions.

Comment.—This case, in which inflamma-

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Fig. 1—Normal lactating mammary tissue along an interlobular duct. Cow 22. x 70.

Fig. 2—Normal wall of a large collecting duct to demonstrate epithelium composed of two layers of cells. Cow 22. x 400.

Fig. 3—Mild hyperplasia of the epithelium of a large collecting duct and infiltration of polymorphonuclear leucocytes into the epithelium. Cow 21. x 400.

Fig. 4—Extensive hyperplasia of epithelium in the wall of a large duct with downgrowths and a roughened surface. Many polymorphonuclear and mononuclear leucocytes appear in the area. Cow 6. x 400.

Fig. 5—Vacuolization of the alveolar epithelium in an area of focal acute inflammation. The lumens are distended with a granular eosinophilic material containing polymorphonuclear leucocytes. Cow 23. x 400.

Fig. 6—Occlusion of a small interlobular duct by a clot containing polymorphonuclear leucocytes and focal infiltration of leucocytes in the surrounding tissue. Cow 22. x 70.

tory lesions were not sufficiently extensive to result in abnormal milk, was characteristic of subclinical streptococcic mastitis.

The gross lesions in the remaining cases were rather similar to those recorded above. To avoid unnecessary duplication, a complete description of each case will not be given but the lesions are summarized and tabulated in detail. The relation of the gross abnormalities to the lesions observed microscopically is summarized in table 1. The incidence of abnormalities in size, color, consistency, and secretion is given for 83 quarters. The numbers of quarters in each group showing a microscopic change is also recorded. There was some correlation between the size of the quarter on gross examination and the lesions observed microscopically. Atrophy on microscopic examination was noted in the majority of quarters, although the proportion was somewhat higher in quarters which were reduced in size. Nearly all quarters which were either enlarged or atrophied showed inflammatory lesions on microscopic examination. All quarters with acute diffuse inflammation were enlarged.

The cut sections were usually a uniform pink which was considered normal color. All of the quarters with abnormal color were inflamed. Most of them also showed atrophy and fibrosis. The latter lesions occurred in quarters which were pink in nearly the same incidence as in quarters with an abnormal color.

Of 29 quarters with a firm consistency on gross examination, atrophy was observed in 27, inflammatory lesions in 28, and fibrosis in only 19. Similar lesions also occurred in the majority of quarters with a spongy consistency, but in a much lower proportion than in the quarters with a firm consistency. The occurrence of abnormal secretion in 25 quarters was usually accompanied by microscopic lesions. In 34 of 50 quarters with a normal secretion, inflammatory lesions were demonstrated.

All four quarters which showed diffuse inflammation on microscopic examination were grossly enlarged, gray or red, and the secretions were abnormal.

The summary in table 1 reveals a relatively slight correlation between gross and microscopic lesions. Definite gross abnormalities could be accounted for by microscopic lesions in every instance except one quarter showing mild atrophy. In the absence of gross abnormalities, however, many quarters showed microscopic lesions. Therefore, although positive findings on gross examination indicated infection, negative gross findings did not exclude it.

As already mentioned, the microscopic lesions in the individual cases resembled those described above. In 3 other instances of acute mastitis similar to cow 20, the diffuse exudative inflammatory process was patchy in extent. The most severe lesions were found near the lactiferous sinus and large ducts. Bacteria were observed mainly in the milk ducts, but a few could be seen in the interstitial tissues and lymph channels.

In chronic mastitis, most inflammatory lesions were focal and varied greatly in size. Some foci involved a few alveoli, whereas others extended over one or more lobules. Some such foci consisted of an exudate of polymorphonuclear leucocytes in the alveoli, with vacuolization of the epithelium. This lesion was designated as acute focal mastitis. Other foci contained shrunken alveoli with interstitial infiltration of lymphocytes, monocytes, and plasma cells, and a variable amount of fibrosis. This lesion was called chronic focal interstitial mastitis.

Perivascular accumulations of lymphocytes and monocytes were observed in some of the areas of interstitial mastitis. These

TABLE 1—The Relation of Gross Appearance to Microscopic Lesions in 83 Quarters

Gross appearance	No. of quarters	No. quarters with microscopic lesions				
		Atrophy	Fibrosis	Focal inflam.	Diffuse inflam.	No lesions
Normal size	54	37	19	31	0	17
Enlarged	9	8	4	5	4	0
Reduced in size	20	20	15	19	0	0
Pink color	71	56	33	47	0	15
Gray color	3	3	3	2	1	0
Red color	9	6	4	6	3	0
Spongy consistency	54	38	21	30	0	16
Firm consistency	29	27	19	24	4	0
Secretion normal	58	41	24	34	0	17
Secretion abnormal	25	21	16	19	4	0

were small, roughly cylindrical, and were associated with thickening of the wall of the arterioles and hyperplasia of the endothelium.

Vacuolization of the epithelium of the alveoli was a very common alteration in the areas of acute inflammation. This alteration was often associated with distended alveoli which contained granular eosinophilic material. An occlusion of a small collecting duct by a clot of fibrin containing leucocytes was observed occasionally. This occlusion was usually associated with an area of inflammation and epithelial vacuolization in adjacent alveoli which were apparently unable to evacuate their secretion because of the occlusion. Occlusion was observed in both intralobular- and interlobular-collecting ducts and smaller ducts were most commonly involved. Most occlusions were of recent origin, although occasionally one was found partially or completely organized. Several organized intraductal occlusions were found to be attached on only part of their length and covered elsewhere by one or two layers of cuboidal epithelium.

Hyperplasia of the epithelium of the larger ducts was commonly found in chronic mastitis. This resulted in outfoldings, pockets, and extremely roughened surfaces. There was often a severe periductal inflammation in the same area.

There was a great variation in the type and severity of lesions in different parts of the same quarter. The ventral portion of most quarters with chronic streptococcic mastitis showed areas of atrophy, fibrosis, and interstitial infiltration of lymphocytes, monocytes, and plasma cells. The middle portion showed less severe atrophy and rarely more than slight fibrosis with interstitial leucocytic infiltration. The dorsal portion of these affected quarters had focal inflammatory areas which were more often characterized by exudation of polymorphonuclear leucocytes into the alveoli and ducts with some interstitial infiltration of monocytes, lymphocytes, eosinophils, and polymorphonuclear leucocytes. In the dorsal portion of the gland, the focal areas of inflammation were usually small and few in number, but more acute.

Normal mammary tissue is shown in figures 1 and 2. Characteristic lesions are illustrated in figures 3 to 11.

CORRELATION OF THE ANATOMIC ALTERATIONS WITH CLINICAL FINDINGS

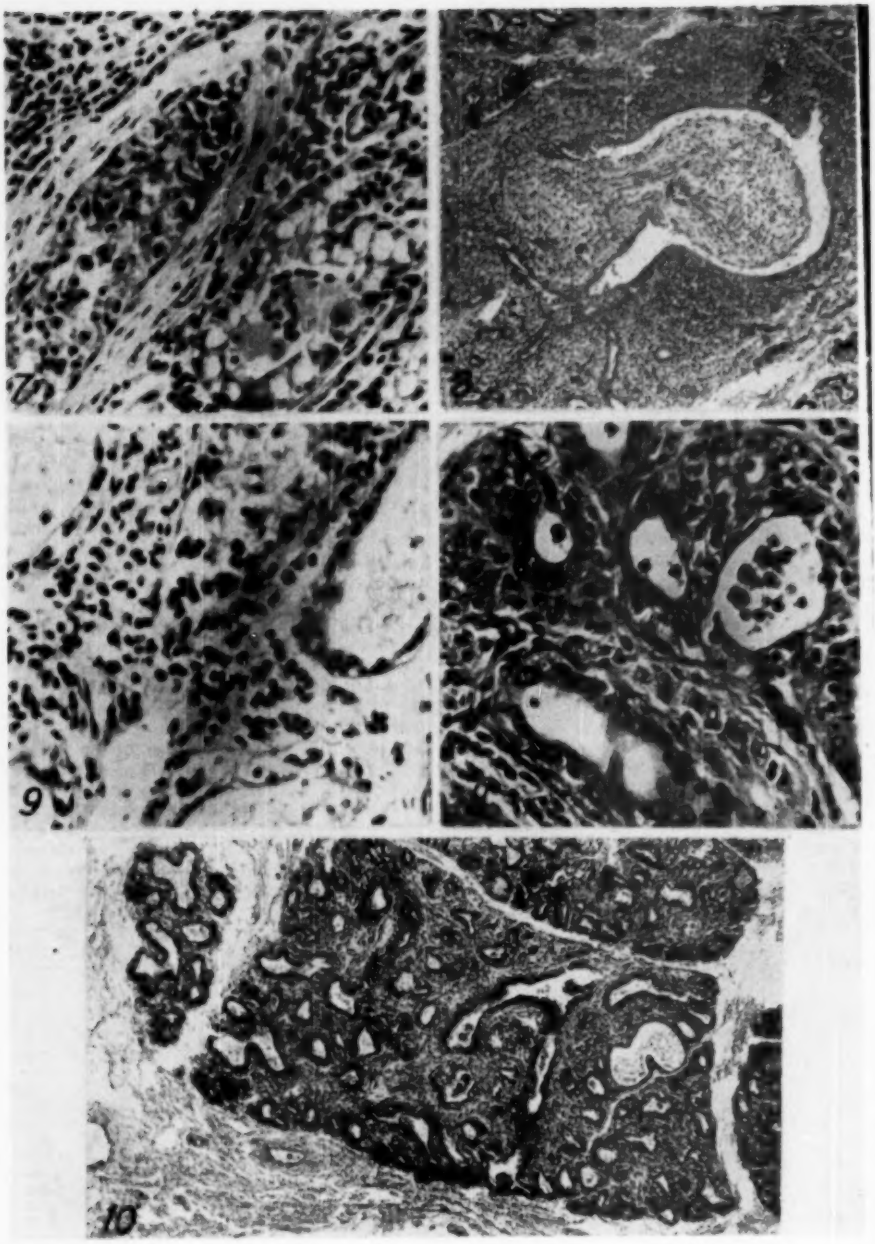
The relation of clinical and bacteriologic findings to the predominant pathologic alterations is recorded in table 2. Three cases of acute mastitis were studied and all showed marked acute diffuse exudative mastitis. In the case infected with *Streptococcus zooepidemicus*, this type of inflammation was predominant. In the other 2 cases, extensive necrosis warranted a diagnosis of acute gangrenous mastitis. Staphylococci were found in 1 case of gangrenous mastitis and a mixed infection of staphylococci, *E. coli*, and *Ps. aeruginosa* were present in the other.

Twenty-two quarters were affected clinically with chronic streptococcic mastitis. These showed a variety of pathologic alterations but chronic interstitial mastitis was the most prominent type of change in 12 quarters. Acute exudative mastitis was the diagnosis for four quarters, and no pathologic alterations were observed in six quarters.

The single quarter affected with chronic

TABLE 2—Relation of Clinical and Bacteriologic Findings to the Pathologic Diagnosis

Clinical type of mastitis	Bacteriologic findings	Pathologic diagnosis	No. of quarters
Acute	*Streptococcus zooepidemicus*	Acute diffuse mastitis	1
	Staphylococci	Acute gangrenous mastitis	1
	Mixed infection	Acute gangrenous mastitis	1
Chronic	*Streptococcus agalactiae*	Normal	6
		Acute focal mastitis	2
		Acute mastitis	1
		Chronic focal interstitial mastitis	9
		Chronic diffuse interstitial mastitis	3
	Staphylococci	Chronic suppurative mastitis	1
	Aerobacter aerogenes	Atrophy	1
		Chronic focal interstitial mastitis	1
None	*Str. agalactiae* (carriers)	Normal	6
		Acute focal mastitis	2
		Chronic focal interstitial mastitis	10
Negative for pathogens		Normal	14
		Atrophy	6
		Acute focal mastitis	1
		Chronic focal interstitial mastitis	3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staphylococcic mastitis showed many abscesses. Since no history was available in this instance, the animal may have been affected with acute staphylococcic mastitis at a previous time. Two quarters were affected with chronic mastitis which was apparently due to *A. aerogenes*. In one of these, no inflammatory changes were observed but atrophy of the parenchyma was prominent. The other quarter showed chronic interstitial mastitis.

With 43 quarters, there was no clinical history of mastitis and the secretion had been chemically normal, so far as could be determined. Of these, 19 eliminated streptococci and, accordingly, were classed clinically as "carriers" of streptococci. No pathologic alterations were observed in six of them. Three showed acute focal mastitis and ten showed chronic focal interstitial mastitis. The focal inflammatory lesions observed in these carrier quarters were very small and often involved only a portion of a lobule. They were very infrequent and, in one quarter, only a single small focus was observed in several sections of tissue taken from different portions of the gland.

In 24 quarters, abnormal milk had never been observed and bacteriologic examinations revealed no pathogenic microörganisms. These apparently normal glands were used for comparisons with quarters known to be affected with mastitis. No inflammatory changes were observed in 20 of these quarters but four had foci of inflammation which resembled those observed in cases of chronic streptococcic mastitis. These lesions may have been due to pathogenic organisms not detected with the bacteriologic methods employed. A few small foci of lymphocytes were observed in the interstitial tissue of all except six quarters. These lymphocytic foci were perivascular and usually contained a few plasma cells and macrophages. They were not considered to be significant.

The frequency and degree of pathologic lesions in quarters infected with streptococci for various time intervals are given in table 3. In the table, the quarters were divided into three groups. There were eight quarters known to have been infected in less than six weeks; 15 infected from six to twenty-six weeks, and 13 infected in more than twenty-six weeks. The exudation of polymorphonuclear leucocytes into milk spaces was rarely more than slight and did not vary significantly with the different durations of infection. Interstitial infiltration of leucocytes was usually found. This, too, varied only slightly with the different durations of infection. Atrophy and fibrosis were found more often and were more severe with increasing duration of infection. A few quarters infected for more than twenty-six weeks showed slight or no fibrosis.

DISCUSSION

Acute Mastitis.—The alterations in acute mastitis indicate that the causative agent spreads rapidly throughout the quarter by way of the duct system. Some invasion of interstitial tissues by the organisms undoubtedly had occurred, since special stains disclosed a few cocci in those areas. Large clumps of cocci were seen in the milk ducts and alveoli; therefore, the milk channels are probably the site of primary multiplication. This observation is strengthened by the fact that most organisms causing acute mastitis grow rapidly in milk and produce large amounts of lactic acid.

The inflammatory irritants in acute mastitis may be the lactic acid, endotoxins or exotoxins, or a combination of these. The large numbers of organisms found in the tissues and milk ducts are sufficient to explain the extensive inflammatory reaction. When the inflammatory reaction once starts, other factors may aid bacterial activity. The medium and small milk ducts were observed to be occluded with masses of fibrin, leucocytes, and organisms. Secretion distended the alveoli in most areas of the gland

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Fig. 7—Chronic interstitial mastitis with infiltration of lymphocytes, monocytes, and polymorphonuclear leucocytes. Vacuolization of the epithelium is marked. Cow 23. x 400.

Fig. 8—Chronic interstitial mastitis with an organized clot in a collecting duct. There is extensive cellular infiltration, fibrosis, and atrophy in the surrounding tissue. Cow 9. x 70.

Fig. 9—A small focus of an early stage of chronic interstitial mastitis in a "carrier" of streptococci. Degeneration of the epithelium is prominent. Cow 22. x 400.

Fig. 10—Marked atrophy and fibrosis in a terminal stage of chronic interstitial mastitis. The alveoli have disappeared, leaving a shrunken lobule containing tubular ducts and many leucocytes. Cow 2. x 70.

Fig. 11—Chronic interstitial mastitis with fibrosis and atrophy. Same section as in figure 10. x 400.

and the epithelium was vacuolated in the same region. Edema of the interstitial tissues appeared to reduce the lumens of the ducts and to increase the tendency toward occlusion. The coagulase produced by staphylococci would also aid coagulation of the exudate in the milk channels.

This damming of the flow of secretion by edema and clots of exudate is probably important in the pathogenesis of acute mastitis because it provides stasis of secretions, thereby favoring enormous multiplication of organisms and consequent increase in the concentration of inflammatory irritants.

Clinical observation of cases of acute mastitis has shown that the disease has a sudden onset associated with conspicuous swelling of the gland and cessation of milk production. The watery yellowish fluid obtained from the teat during the early stage of the disease often forms a clot after it is drawn. It is probable that some milk is being produced at this early stage but it cannot flow downward because of occlusion of the ducts. The second or third day of the disease is usually marked by an increased amount of secretion which contains numerous clots varying in size from microscopic to several millimeters in diameter. The appearance of large numbers of clots in the secretion and a softening of the gland is evidence of a regression of the process. Contraction of clots may aid in their expulsion on the second or third day of the inflammatory process.

Chronic Streptococcic Mastitis. — The most constant characteristic of the lesions of chronic streptococcic mastitis was the scattered distribution of foci of inflammation. It is probable that many of these foci were related to a partial or complete blocking of small ducts by fibrin clots. Evidence of this hypothesis was as follows:

1) The alveoli in the foci of inflammation were dilated.

2) The alveolar epithelium in the inflammatory foci was vacuolated. Observation of the mammary glands of rabbits and guinea pigs in other experiments indicated that this alteration was caused by a failure of removal of the secretion for one to two days.

3) Clots of exudate were observed occasionally in an occluding position.

The inflammatory foci appeared to have been formed at irregular intervals since some foci showed only polymorphonuclear leucocytes, whereas others were of long duration, as evidenced by the presence of fibrosis and atrophy. Quarters in which the approximate duration of infection was less than six weeks showed lesions similar to quarters infected for six months or longer. Foci of acute inflammation were observed in most quarters with chronic streptococcic mastitis of long duration. Apparently, the process of development of lesions is a periodic extension to new areas as the older areas undergo atrophy.

Considerable difference was found in the lesions at different levels in the gland. The dorsal portions contained few inflammatory foci and those present were small with fewer chronic alterations such as atrophy and fibrosis. The ventral portions of quarters were more severely affected and a considerable amount of atrophy and fibrosis was observed. This greater severity of lesions in the ventral portion of the gland is probably due to the more constant exposure of that part by streptococci released into the milk channels from active foci.

The relation of clinical findings to the gross and microscopic lesions was of much practical interest. Quarters which were clinically and bacteriologically normal were spongy, pink, and of a normal size in comparison with the other quarters of the same cow. Of 19 quarters which were eliminating streptococci, but producing otherwise nor-

TABLE 3—Frequency and Degree of Pathologic Alterations in Quarters Infected with Streptococci for Different Time Intervals

Pathologic alteration	Duration infection (weeks)	No. quarters showing lesion to the following degree:			
		Absent	Slight	Moderate	Marked
Exudation of polymorphonuclear leucocytes into alveoli	Under 6	3	2	0	2
	6 to 26	9	6	0	0
	Over 26	3	7	2	1
Interstitial infiltration of leucocytes	Under 6	1	3	3	1
	6 to 26	0	12	2	1
	Over 26	1	6	2	4
Atrophy	Under 6	4	4	0	0
	6 to 26	0	5	10	0
	Over 26	1	3	5	4
Fibrosis	Under 6	7	1	0	0
	6 to 26	9	4	2	0
	Over 26	3	4	4	2

mal milk, 16 were also spongy, pink, and normal in size. In other words, gross examination failed to disclose lesions in carriers of streptococci. In 50 quarters having a spongy consistency, microscopic lesions of mastitis were found in 30. These findings indicate that a streptococcic infection usually produces microscopic evidence of mastitis, but often does not result in grossly visible lesions.

Inflammatory lesions were observed in 13 of 19 carrier quarters. It is possible that a more extensive examination would have disclosed lesions in the remaining six quarters. From the standpoint of pathology, most cases of streptococcic infection of the mammary gland are really streptococcic mastitis.

Murphy[19] has proposed a three-phase concept of development of mastitis involving invasion, infection, and inflammation. The results reported here do not support such a separation because the inflammatory lesions were found as early as two weeks following the first discovery of streptococci in the milk. Acute inflammatory lesions were similar in recently infected quarters to those infected for many months. It is probable that focal inflammation develops within a few days after streptococci enter an area. Until a sufficiently large proportion of the gland is involved, the exudate from inflamed areas would not create a noticeable abnormality when diluted with normal milk.

Entrance of organisms into the teat ordinarily precedes invasion of the gland. The interval between those two processes might be hours, days, or weeks. An exact division between invasion and infection would appear difficult and of little practical importance. From a clinical standpoint, the occurrence of *Str. agalactiæ* in the milk is good evidence that both infection and inflammation are present in the gland. The results reported here indicate that inflammation of the gland is not only caused by and accompanies infection, but it probably also aids the infection by hindering the normal flushing of milk flow.

The inflammation appeared more severe than would be expected from the small number of organisms found on quantitative culture or direct microscopic examination of stained tissues. Since *Str. agalactiæ* has not been shown to produce highly toxic materials, it probably injures the mammary tissues in a manner not yet understood. The inflammatory irritant produced by the organism might be lactic acid, although the small numbers of organisms present and the natural buffering materials in milk make that possibility unlikely. The milk rarely shows an increased acidity during the early stages of an exacerbation of inflammation. The lesions and clinical characteristics of chronic streptococcic mastitis indicate that some cows are much more susceptible to the organisms, so that small numbers of bacteria can initiate a severe inflammatory reaction.

The classification and nomenclature of mastitis has been in a confused state because of the variety of terms employed and the lack of clinical correlation. The division of mastitis into acute and chronic is well founded from the standpoint of clinical and postmortem studies. The term parenchymatous mastitis seems to have little value because the parenchyma of the organ is always primarily involved in any inflammatory process. The term catarrhal mastitis is unsatisfactory because the lining of the ducts is not a mucous membrane and no mucus has been demonstrated in the secretions. The terms interstitial mastitis and exudative mastitis have been used to designate the predominate type and location of the inflammatory exudate and are probably of considerable value in description of the lesions. They are of little value from a clinical standpoint because many of the cases described in this study showed both types of lesions in different parts of the same gland. The term atrophy is applicable to both clinical and postmortem findings. The use of the term fibrosis in relation to firm areas found on palpation is inadvisable because, in this study, such areas were often found to be regions of interstitial edema and retained secretions. Fibrosis was difficult to detect on palpation and it was found to accompany atrophic lesions of long duration.

From the clinical standpoint, an etiologic designation for the type of mastitis is preferable together with the terms acute or chronic. The phrase "with atrophy" may be added where indicated.

The roughening of the surface of the milk ducts as a result of hyperplasia of the epithelium was suggested by Johnston[20] to be an important factor in the pathogenesis of bovine mastitis. This lesion was found in many of those cases of chronic streptococcic mastitis described here. Such roughening and pocketing would hinder the flushing action of the milk in removal of microorganisms at milking time.

The principal site of persistence and multiplication of streptococci is probably in the ventral portion of the gland on the epithelium of the ducts and lactiferous sinus. The ease with which many streptococcic infections can be eradicated by antibacterial therapeutic agents is further evidence that the usual site of the organisms is the ventral portion of the gland.

SUMMARY

The pathologic alterations in the mammary glands of 25 dairy cows were correlated with the antemortem, clinical, and bacteriologic findings. In cases of acute mastitis, there was an acute diffuse exudative inflammation, and sometimes extensive necrosis. Mammary glands affected clinically with chronic streptococcic mastitis showed both chronic interstitial and acute focal inflammation. Most of the clinically normal animals which were eliminating streptococci also had focal lesions of chronic interstitial and acute mastitis. Many such glands with no gross abnormalities showed microscopic evidence of inflammation.

Lesions of various ages were present in most affected glands, and there was a gradual progression toward atrophy and fibrosis. The lesions were more extensive and severe in the ventral than in the dorsal portion of the glands. These results indicate that a streptococcic infection usually causes progressive inflammatory lesions in the gland.

The roughening of the surface of the epithelium of the milk ducts and the occlusion of ducts by clots of fibrin are probably important in the pathogenesis of bovine mastitis.

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The *Brucella Abortus* Ring Test

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THE RING TEST for brucellosis was first described by Fleischhauer[1] and Fleischhauer and Hermann,[2] in 1937 and 1938. The new test for the detection of Brucella agglutinins in whole milk was found to be sufficiently sensitive to be applied to pooled milk samples from a herd. This fact permitted the test to be used on pooled or mixed milk samples, from a given herd, collected at the receiving line in a milk-processing plant. It has thus served as a simple and rapid means of locating Brucella-infected herds.

In a high percentage of cases, positive results were obtained even though the pooled sample tested represented the milk from 1 infected animal mixed with the milk from 5 to 15 noninfected cows.

The ring test is actually an agglutination test and depends on the same basic principle as the blood serum-agglutination test. The agglutinins or antibodies which the infected animal develops in attempting to rid itself of the disease are usually found in the milk as well as in the blood. The antigen used (3 to 4% *Brucella abortus* cells) is stained a deep blue color with hematoxylin so that the agglutination reaction may be observed in the presence of whole milk.

The test is conducted by adding 1 drop of stained antigen to 1 cc. of milk in a small test tube (10 mm. or less, inside diameter). After the sample is thoroughly mixed, it is incubated at 37 C. for forty-five to fifty minutes or left at room temperature for sixty to ninety minutes. This is sufficient time for the cream line or ring to form.

In milk from an infected herd, the stained antigen agglutinates and collects on the fat droplets. As the cream rises to the top, a deep blue cream line or ring is formed. The skimmilk fraction, therefore, is white, in strongly positive samples. In milk from negative herds, the antigen remains in the skimmilk fraction, giving it a light blue color; the cream ring is white. The nature of the reaction observed gives the test its name. In Europe, the designation "ABR test" (Abortus Bang Ring test) has been applied.

Since the original description of the test by Fleischhauer, a number of studies have been reported, particularly from Denmark and Sweden.[3-9] These reports described improved methods of preparing the antigen, technique of conducting the test, and rather extensive studies showing the efficiency of the test in detecting infected herds. It is now an official adjunct to the blood-agglutination test in the brucellosis control programs of these countries.

As the milk is received at the dairy plant, small samples from each are obtained. Herds showing positive ring tests are then subjected to the blood test to locate infected or reactor animals.

In Denmark for example, a herd may be accredited as brucellosis free on the basis of one negative blood test, provided the herd has had two negative ring tests at three- to six-month intervals preceding the blood test. This procedure saves considerable time and expense.

Some idea of the efficiency of the ring test in locating infected herds was presented in the reports of Seit and Jorgensen,[5] and of Christiansen,[9] working in Denmark. These investigators conducted ring tests on a total of 12,605 herds. Of this number, 2,031 were classed as infected or reactor herds on the basis of the blood test. The ring test was positive on 1,665 (82%) of the reactor herds. Reactor herds negative to the ring test which were investigated failed to agree in about two thirds of the cases, because the reactor animals present were not in production. That is, heifers, dry cows or, in some instances, a bull or steer were the reactors. Usually, only 1

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such reactor animal was found per herd. It was considered likely that the majority of such herds would be detected on a second ring test in three to six months.

Nonproducing reactors represent a natural limitation of a single ring test. The workers cited believe this limitation to be correctable in large part by ring testing twice a year.

The reports from the Scandinavian countries were so encouraging that the ring test was considered for use in the brucellosis control program in those counties in Minnesota which are under the area control plan. In coöperation with the U. S. Bureau of Animal Industry, and the State Livestock Sanitary Board, studies on the ring test under field conditions were initiated in the fall of 1947.

Ring tests were made in as many counties as possible just preceding the county-wide blood tests for reaccreditation of the counties. This procedure permitted a comparison of the results of the ring tests with those of the blood tests. A preliminary report[10] has been given of the results obtained in seven such counties studied. This preliminary report included a study of 6,116 herds, of which 290 were classified as infected or reactor herds on the basis of the individual animal blood tests. The ring test was positive on 217 (75%) of these reactor herds. Fifty-six per cent of the failures (reactor herds missed by the ring test) was due to nonproducing reactors in the herds.

The present report is a summary of a total of 30,811 ring tests of herds in 25 counties in Minnesota on the area control plan. In nine of the counties, the ring tests preceded county-wide blood tests. In the remainder of the counties, blood tests were conducted only in those herds which were positive to the ring test.

METHODS

Preparation of the Antigen.—The stained antigen was prepared either from the U. S. Bureau of Animal Industry stock *Br. abortus* tube or the plate antigen. The antigen was stained with oxidized hematoxylin in the presence of ammonia aluminum sulfate as a mordant. The excess dye was removed by centrifugation and washing with a dilute sodium chloride solution adjusted to pH 4.0 with hydrochloric acid. The stained cells were suspended in a 50-50 mixture of glycerin and physiologic saline containing 0.5 per cent phenol. The final concentration was 4 per cent Brucella cells.

The method of preparing the antigen was,

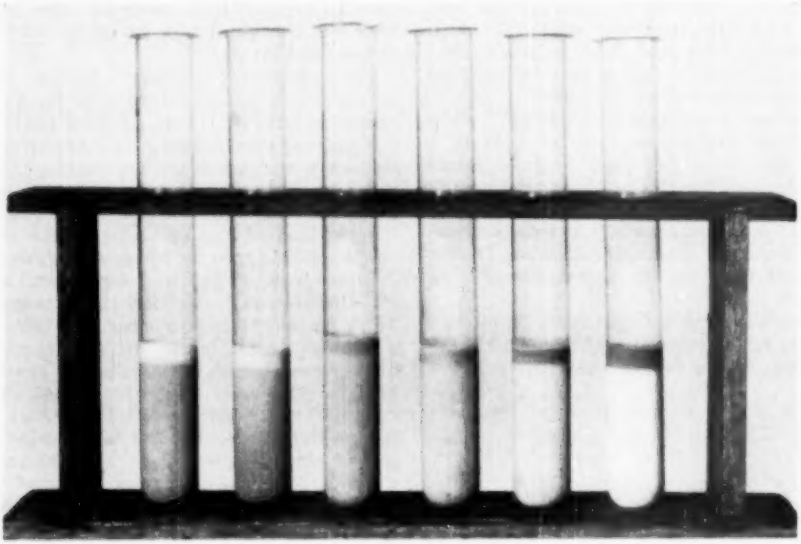

Fig. 1—Milk ring tests. From left to right: tubes 1 and 2, negative; tubes 3 and 4, + + +; tubes 5 and 6, + + + + reactions.

with slight modification, that given in detail in the previous publication.[18]

Collection of Milk and Cream Specimens.—Small test tubes (12.5 by 100 mm.) were used for the collection of the milk and cream samples. The milk or cream specimens were identified by the patron number assigned to the herd owner by the creamery, cream-buying station, or dairy plant.

In the early part of the studies, only one milk sample was taken for each herd from the weighing tank on the milk-receiving line of the dairy plant. This procedure was soon discontinued when evidence was obtained of errors in the results of the test, due to contamination of the milk from the small amount of milk remaining in the weighing tank from the preceding herd. In over three fourths of the ring tests on milk, pooled samples were obtained on the receiving line by taking about 3 ml. of milk from each of two or three cans of milk from a given herd. This procedure usually did not involve the collection of more than two or three such pooled samples per herd, since most of the dairy herds in the areas concerned in this study were small. The average daily production per herd was from four to six cans of milk per day with a low percentage of the herds producing more than nine cans per day. In a few herds, samples were taken from each can.

Cream specimens (4 to 6 ml.) were collected at the time the creamery operator secured a sample of cream from each can for butterfat determination, or the cream samples were taken later from vials containing cream saved for such butterfat analysis.

Two drops of a 1:12 dilution of formalin were added to each collecting vial as an aid in preserving the milk or cream specimens. After milk or cream specimens were collected from the majority of herds supplying a given plant, empty vials containing the preservative were left with the operator for collection of specimens from herds which had been missed. These latter specimens were placed in a cooler until they could be picked up a day or two later for testing.

The ring tests were conducted in a portable laboratory, centrally located from the standpoint of creameries and dairy plants in the area under study. In some instances, the portable laboratory was housed in a trailer; in other instances, suitable facilities were arranged in the county courthouse or a city hall.

Technique of Conducting the Ring Test.—The test was performed by pouring 2 ml. (estimated) of the milk specimen, which had been thoroughly mixed, into a small test tube (10 by 100 mm., inside dimensions). Two drops of the antigen were added, the sample thoroughly but gently mixed by inverting the tube several times, then left at room temperature for seventy-five to ninety minutes before reading the results.

A fairly high percentage of Minnesota dairymen market only cream. It was thus necessary to modify the test to render it applicable to cream. The test on cream was performed by adding to a small test tube 1 ml. of physiologic saline, 2 drops of a saturated sodium bicarbonate solution to neutralize any acid which might be present in the cream, 12 drops of the cream specimen, and 1 drop of the antigen. The samples were mixed thoroughly, let stand at room temperature, and the results noted as for the test on milk.

Arbitrary designations were used to indicate the degree of reaction to the ring test. These ranged from negative to four plus. The tests were read as negative, if the cream line or ring were white, or were lighter in color than the skimmilk fraction; one plus, if the blue color of the cream ring were equal to that of the skimmilk fraction; two plus, if the

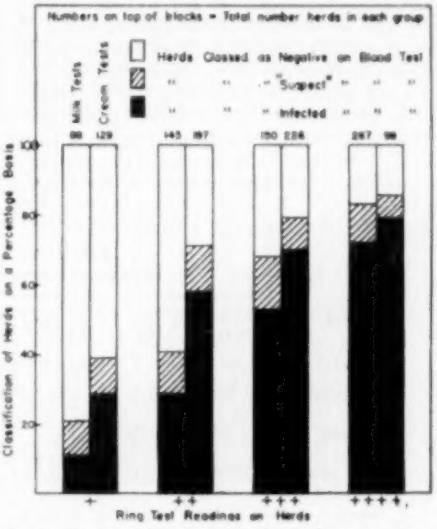

Fig. 2—Results of blood tests on herds showing various degrees of reactions to the ring tests.

color of the cream ring were slightly darker blue than the skimmilk fraction; three plus, if the color of the cream ring were markedly bluer than the skimmilk fraction; and four plus, if the color of the cream ring were a very dark blue and the skimmilk fraction essentially white (indicating that nearly all of the stained antigen was in the cream ring).

Figure 1 illustrates the ring test and shows some of the variations and results obtained

with negative and positive milk samples. With the exception of those herds in which it was known that reactor animals were being retained, blood tests were made, usually within one month, in all but 11 herds positive to the ring test. The blood test was considered as the standard, from the standpoint of the determination of the accuracy and efficiency of the ring test in detecting Brucella-infected herds.

RESULTS

Table 1 is a summary of the results obtained in nine counties in which county-wide blood tests were conducted within one to two months following the ring tests. With the exception of St. Louis County, the majority of the tests in the different counties were on cream specimens.

The over-all agreement of 96.2 per cent, obtained in the comparison of the two tests, would seem on first thought quite good. However, it should be noted that of the 8,469 herds involved in the study, only 385 (4.5%) were classified as infected on the basis of the blood test. Had the ring test been negative throughout, over-all agreement could have been considered as 95.5 per cent.

Of particular interest in this study was the efficiency of the ring test in detecting infected herds. That is, the percentage of infected herds on the basis of the blood test, which was positive to the ring test, constituted a measure of the efficiency of the latter. These values for the different counties, and the over-all average, are given in the right-hand column of table 1.

In Koochiching, Itasca, St. Louis, Hubbard, Kittson, and Roseau counties the studies were made during late summer and fall, when the milk production was at a low level and a relatively high percentage of the cows was not in production. Of the 121 infected herds which were negative to the ring test, 107 were investigated to determine possible causes for the negative results. Of the 107 failures to detect the infected herds, 69 (64.5%) were due to non-production, at the time of the test, on the part of the only reactors present in the herds. Usually, only 1 such reactor animal was present per herd. In several additional instances, the milk from the reactor animal was saved for family use, or the animal was a nurse cow for the calves. In one instance, the reactor was the herd sire.

TABLE 1—Comparison of Results of Blood Tests with Herd Ring Tests in Counties with County-Wide Blood Tests

County	No. Herds Ring Tested	Agreement of Ring and Blood Tests			Disagreement		Percent Efficiency In Locating Inf. Herds
		Blood − Ring −	Blood + Ring +	Percent Agreem't	Blood + Ring −	Blood − Ring +	
Koochiching	228	210	11	96.0	5	2	69
Itasca	584	573	4	98.8	2	5	67
St. Louis	2,704	2,502	61	94.7	20	121	75
Lake	49	48	1	100.0	0	0	100
Watonwan	828	748	56	97.8	15	9	79
Wilkin	669	558	69	94.8	15	26	82
Hubbard	1,054	1,014	15	98.5	15	10	50
Kitson	990	906	33	94.9	39	12	46
Roseau	1,363	1,326	14	98.1	10	13	58
Total	8,469	7,885	264	96.2	121	198	68

The number of herds positive to the ring test but negative to the blood test (false positive ring test) was unusually high in St. Louis County. This was considered as being due to the method of collecting the milk samples (from the weighing tank) and to the fact that a large amount of partially frozen milk was received at the dairy plants during the period of study in the county. The collection of "iced milk" on the screens over the weighing tank materially increased the chance of contaminating the succeeding lot of milk weighed. It was also found that negative milk which has been frozen frequently gives a false reaction (+ or ++) to the ring test.

The results of the blood test in 1,300 herds positive to the ring test (650 positive to the ring test on cream, and 650 positive to the herd tests on milk) are shown in table 2 and figure 2.

The herds were classified, on the basis of the blood test, as follows: infected, if 1 or more animals in the herd were positive to the blood test; suspicious, if 1 or more animals showed a suspect titer, but all other animals were negative; negative, if all animals in the herd were negative to the blood test.

Over-all agreement of the two tests of 70 per cent for the positive ring tests on cream specimens and 62 per cent for the positive ring tests on milk was found. In so designating the agreement, it was considered that there was agreement between the two tests even though some herds were classified as suspicious on the basis of the blood test. As might be expected, the percentage of herds classified as reactor herds was highest for those herds in which three or four plus ring reactions were obtained.

The fact that only 11 per cent of 88 herds with one plus milk tests were infected, as indicated by the blood test, suggests that one plus milk reactions, as obtained under the conditions of this study, might well be disregarded from the standpoint of follow-up blood tests in routine field use of the ring test. Particularly might this hold if reactions in the strong one plus range were read as two plus reactions. It would seem likely that the occasional infected herd which might be missed by this procedure would be detected by subsequent ring tests.

For the two to four plus milk reactions, herd classification on a blood test basis was: 56 per cent infected, 12 per cent suspicious, and 32 per cent negative. This constitutes an over-all agreement of 68 per cent. From the standpoint of the sensitivity of the ring test on cream versus that on milk, a one plus reaction on cream appeared equivalent to a two plus reaction on milk; likewise, a two plus reaction on cream appeared equivalent

TABLE 2—Results of Blood-Agglutination Tests on Herds Positive to the Milk or Cream Ring Tests for Brucellosis

Comparisons	Results of Blood Tests on Herds Positive to Ring Tests												Overall Agreement			Total No. Herds
	Positive Herd Cream Tests															
	129 + Tests			197 ++			226 +++			98 ++++						
	R*	S*	N*	R	S	N	R	S	N	R	S	N	R	S	N	
Number of Herds	38	13	78	115	25	57	159	20	47	77	7	14	385	65	96	650
Percent of Herds	29	10	60	58	13	29	70	9	21	79	7	14	60 (70)	10	30	
	Positive Herd Milk Tests															
	88 + Tests			145 ++			150 +++			267 ++++						
Number of Herds	10	9	69	42	18	85	75	23	48	193	28	46	324	78	248	650
Percent of Herds	11	10	78	29	12	59	53	15	32	72	11	17	50 (62)	12	38	

R = Herds with one or more animals positive to blood test
S = Herds with one or more animals giving "suspect" titers but no reactors
N = Herds negative to blood test

to a three plus reaction on milk, etc., (*see* fig. 2).

Another point of interest is that the percentage of herds classified as suspicious on a blood test basis was relatively constant, irrespective of the degree of reaction to the ring test (+ to ++++), on either milk or cream.

The relatively high percentage of false positive ring tests (*i.e.*, herds positive to the ring test but negative to the blood test) obtained in this study might seem objectionable from the standpoint of routine field use of the ring test as an adjunct to the blood test. The percentage of false positive ring tests could be reduced either by reducing the sensitivity of the antigen or by disregarding the weaker (+ to ++) reactions. However, in either case, an appreciable percentage of the infected herds was missed. This would defeat the main purpose of the use of the ring test as a "screening" or presumptive test.

A very limited amount of information was obtained which indicated that, for a significant percentage of the ring tests designated as false positive, the tests actually were not in error. In one county in which two county-wide ring tests were conducted six months apart, the first test showed 37 false positive tests. Six months later, both the ring and blood test results were positive for six of the 37 herds. In these six herds, the herd ring test detected the infection ahead of the individual animal blood tests.

DISCUSSION

In the comparisons made of the ring and blood tests, all discrepancies were charged as errors against the ring test. Some of the errors rightfully should not have been so charged. These errors included: herds which became infected during the interval between the two tests, inherent inaccuracies of the blood test, instances in which the ring tests were positive before the blood tests indicated infection, and inaccuracies which may have occurred during the period of training personnel in the technique of conducting the test.

In terms of simple approximate ratios, the results of the ring test may be summarized as follows: The ring test was positive on two out of three herds classed as infected on the basis of the blood test results; nine out of ten herds in which reactor animals were in production were positive to the ring test; two out of three of the failures of the ring test to detect reactor herds were due to the fact that the infected animals in the herds were not in production; and two out of three herds positive to the ring test were classed as reactor or as suspicious herds on the basis of the blood test results.

When reasonable efforts were made to collect as many specimens of milk or cream as possible over a period of a week at creameries, cream-buying stations, and dairy plants, specimens were obtained from 80 to 90 per cent of the herds, in predominately dairy counties. In the western part of the state where beef herds were more numerous, the percentage was as low as 60.

The results of the study indicate that in counties where dairying is predominant, one county-wide ring test may be expected to serve as a means of locating 50 to 60 per cent of the infected herds, and two tests six months apart to disclose from 75 to 80 per cent of the reactor herds.

In counties under the area control plan in Minnesota, the percentage of infected herds is low. To keep the infection under control, it is necessary to conduct blood tests on 20 to 100 herds to find one infected herd. Thus, the time and expense of locating an infected herd with the blood test alone are quite great.

It is estimated that the cost of a county-wide ring test is about 10 per cent of that of a county-wide blood test. If used regularly once or twice a year in the counties under the area plan, the ring test would make fewer county-wide blood tests necessary and at the same time provide a means of holding the infection at a lower level than would be possible with blood tests alone at three-year intervals.

The reports by Bruhn[7] and by Christiansen[8] on the results of simultaneous blood and ring tests on individual animals suggest that the ring test is of limited value on an individual animal basis. They found a relatively high percentage (5 to 10%) of positive ring tests on animals which were negative to the blood test. For the same reason, it would seem that the test would not be satisfactory for use by individual herd owners. Such use of the test might cause a great deal of confusion. Because the ring test is not highly accurate and by itself does not furnish final proof that an animal

or a herd is infected, positive ring tests should be followed up with blood tests.

The ring test would probably be used best in an official type of coöperative program which involves the coöperation of the dairymen, creamery, and milk-producing organizations and the animal disease-control agencies. Such a program should provide for follow-up blood tests on herds showing positive ring tests and provide help to owners with infected herds.

SUMMARY

Ring tests were conducted on herd milk or cream specimens collected at creameries or dairy plants in 25 counties under the area brucellosis control plan. A total of 30,811 herd tests were made. Comparisons of the results of the ring tests with the results of the blood tests were as follows:

1) In a comparison of the ring and blood tests on 8,469 herds (4.5% infected), there was an agreement between the two tests on 96.2 per cent of the herds.

2) The ring test was positive for 264 (68%) of 385 herds which were considered as being infected on the basis of the blood test for brucellosis.

3) Investigations in 107 infected herds which were negative to the ring test gave the information that in 69 herds (65%) the reactor animals were not in production.

4) The ring test was positive for 88 per cent of the herds in which infected animals were in production.

5) Blood tests on 650 herds which were positive to the ring test showed 56 per cent to have 1 or more infected animals present, 12 per cent to have 1 or more suspicious animals, and 32 per cent to be negative.

6) Of 650 herds positive to the ring test on cream, 60 per cent were classed as infected, 10 per cent as suspicious, and 30 per cent as negative to the blood test.

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Report on Subtilin and Bacitracin as Possible Treatment for Bovine Mastitis

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SINCE 1939, when the infusion of chemotherapeutic agents into the bovine udder as a means of treating mastitis first received serious attention in this country, a considerable number of agents have been tested as possible treatments for this disease. Many of these agents have proved to be effective in varying degrees but all have had certain drawbacks or limitations, such as being irritating to mammary tissue, being difficult to administer, having a limited antibacterial spectrum, or requiring repeated infusions to effect bacteriologic cures. The desirability of having an agent suitable for infusion into the bovine udder which has a wider spectrum of antibacterial activity and which would require a smaller total number of infusions than the agents now available for treating mastitis is self evident. For this reason, the testing of new antibiotics as possible treatments for this disease has been continued.

This report deals with two such antibiotics obtained from cultures of specific strains of *Bacillus subtilis*, subtilin[1] and bacitracin.[2] It had been shown that subtilin was active chiefly against gram-positive bacteria[3] and had an extremely low toxicity for embryonic tissue, being approximately 20 times more toxic to *Staphylococcus aureus* than to chick-heart tissue.[4] Bacitracin had also been found to have certain properties,[2] such as being neutral, nontoxic, water soluble, being active against gram-positive bacteria, and having a wide antibacterial spectrum which suggested that it might prove to be an effective agent for treating mastitis.

TABLE I—Concentration of Subtilin in Blood Agar Inhibiting Growth of Microörganisms Associated with Mastitis

Culture type	No. of cultures	μg. subtilin per cc. of agar						
		Control	0.7	1.4	2.9	5.9	11.9	23.8
	3	+ + + +	.....	.....	.....	.....	.....	.....
	1	+ + + +	+	.....	.....	.....	.....	.....
Streptococcus agalactiae	1	+ + + +	+ +	+	.....	.....	.....	.....
	9	+ + + +	+ + + +	+ + + +	+	.....	.....	.....
	16	+ + + +	+ + + +	+ + + +	+ + + +	+ +	.....	.....
	2	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+	.....
	1	+ + + +	+ + + +	+ + + +	+ +	+ +	+ +	+
Streptococcus uberis	8	+ + + +	+	.....	.....	.....	.....	.....
	2	+ + + +	+ +	+	.....	.....	.....	.....
	10	+ + + +	+	.....	.....	.....	.....	.....
Hemolytic staphylococci	14	+ + + +	+ +	+	.....	.....	.....	.....
	2	+ + + +	+ + +	+ +	+	.....	.....	.....
	4	+ + + +	+ + + +	+ + + +	+ +	+	.....	.....
	3	+ + + +	+ + + +	+ + + +	+ + + +	+ + +	+	.....

..... = no growth; + = slight growth; ++ = growth half as dense as on control plate; +++ = growth slightly less than on control plate; ++++ = growth comparable to that on control plate.

The subtilin for this work was supplied by the Bureau of Agricultural and Industrial Chemistry, Western Regional Research Laboratory, Albany, Calif., in two lots, 122-V-1 and 200A. The bacitracin was supplied by the Commercial Solvents Corporation, Terre Haute, Ind. This material was of lot No. B-480224, having a potency of 46 units per milligram.

IN VITRO STUDIES

The same procedure was followed, in testing subtilin and bacitracin for their activity against microörganisms commonly associated

The part of this project dealing with subtilin was performed in coöperation with the Western Regional Research Laboratory, Bureau of Agricultural and Industrial Chemistry, U. S. Department of Agriculture, Albany, Calif.

From the Animal Disease Station, Pathological Division, Bureau of Animal Industry, U. S. Department of Agriculture, Beltsville, Md.

TABLE 2—Concentration of Bacitracin in Blood Agar Inhibiting Growth of Microörganisms Associated with Mastitis

Culture type	No. of cultures	Control	Units bacitracin per cc. of agar					
			0.062	0.125	0.25	0.5	1.0	2.0
Streptococcus agalactiae	31	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
Streptococcus uberis	1	+ + + +	+ +	.....	.....	.....	.....	.....
	3	+ + + +	+ + + +	+ + +	+	.....	.....	.....
	1	+ + + +	+ + + +	+ + + +	++	+	.....	.....
	1	+ + + +	+ + + +	+ + + +	+ + + +	+ + +	+	.....
	3	+ + + +	+ + + +	+ + + +	+ +	.....	.....	.....
Hemolytic staphylococci	9	+ + + +	+ + + +	+ + + +	+ + + +	+ +	.....	.....
	2	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	.....	.....
	3	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+	.....
	2	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + +	+ +

..... = no growth; + = slight growth; ++ = growth half as dense as on control plate; +++ = growth slightly less than on control plate; ++++ = growth comparable to that on control plate.

TABLE 3—Concentration of Bacitracin and Penicillin in Blood Agar Inhibiting the Growth of Microörganisms Associated with Mastitis

Culture type	No. of cultures	Control	Units of penicillin and bacitracin per cc. of agar					
			⌘.00195 x.0078	.0039 .0156	.0078 .0312	.0156 .0625	.0312 .1250	.0625 .2500
(x) *Streptococcus agalactiae*	21	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + +	.....
Streptococcus uberis	2	+ + + +	+ + + +	+ + + +	+ + +	.....	.....	.....
Hemolytic staphylococci	11	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ +	.....

⌘ = dilution of penicillin; x = dilution of bacitracin; (x) = these strains of *Streptococcus agalactiae* did not ferment trehalose; ..... = no growth; + = slight growth; ++ = growth half as dense as on control plate; +++ = growth slightly less than on control plate; ++++ = growth comparable to that on control plate.

TABLE 4—Results of Treating Infected Udder Quarters with Subtilin

Cow	Quarter	Type of infection	Treatment			Results of treatment
			1st dose	Interval	2nd dose	
1	L.F.	*Str. dysgalactiae*	0.20 Gm.	.....	.....	Infection not eliminated
	R.R.	*Str. agalactiae*	0.20 Gm.	.....	.....	Infection eliminated
	L.R.	*Str. dysgalactiae*	0.20 Gm.	.....	.....	Infection not eliminated
2	R.F.	*Str. agalactiae*	0.10 Gm.	24 hr.	0.05 Gm.	Infection eliminated
	R.R.	Staph.	0.10 Gm.	24 hr.	0.05 Gm.	Infection not eliminated
3	R.F.	*Str. agalactiae*	0.10 Gm.	24 hr.	0.05 Gm.	Infection not eliminated
	L.F.	*Str. agalactiae*	0.10 Gm.	24 hr.	0.05 Gm.	Infection not eliminated
	R.R.	*Str. agalactiae*	0.10 Gm.	24 hr.	0.05 Gm.	Infection not eliminated
	L.R.	*Str. agalactiae*	0.10 Gm.	24 hr.	0.05 Gm.	Infection not eliminated
4	R.F.	*Str. agalactiae*	0.10 Gm.	24 hr.	0.05 Gm.	Infection not eliminated
	L.F.	*Str. agalactiae*	0.10 Gm.	24 hr.	0.05 Gm.	Infection not eliminated
	R.R.	*Str. agalactiae*	0.10 Gm.	24 hr.	0.05 Gm.	Infection not eliminated
	L.F.	*Str. agalactiae*	0.10 Gm.	24 hr.	0.05 Gm.	Infection not eliminated
5	R.F.	Staph.	0.10 Gm.	24 hr.	0.10 Gm.	Infection not eliminated
	L.F.	Staph.	0.10 Gm.	24 hr.	0.10 Gm.	Infection not eliminated
	L.R.	Staph.	0.10 Gm.	24 hr.	0.10 Gm.	Infection not eliminated
x6	R.F.	*Str. agalactiae*	0.10 Gm.	40 days	0.20 Gm.	Infection not eliminated
	L.F.	*Str. dysgalactiae*	0.10 Gm.	40 days	0.20 Gm.	Infection not eliminated
x6	R.F.	*Str. agalactiae*	0.20 Gm.	40 days	0.20 Gm.	Infection not eliminated
	L.F.	*Str. agalactiae*	0.20 Gm.	40 days	0.20 Gm.	Infection not eliminated
	R.R.	*Str. agalactiae*	0.20 Gm.	40 days	0.20 Gm.	Infection not eliminated
	L.F.	*Str. agalactiae*	0.20 Gm.	40 days	0.20 Gm.	Infection not eliminated

x = cows treated during dry period; R.F. = right front quarter; L.F. = left front quarter; R.R. = right rear quarter; L.R. = left rear quarter; Staph. = hemolytic staphylococci.

with bovine mastitis, as was used in testing like organisms for their sensitivity to the action of penicillin.[5] In brief, this procedure consisted in streaking a standardized loopful of a suspension of each culture on a series of blood agar plates, each plate of the series containing a definite concentration of the antibiotic in the medium. The cultures of microorganisms were isolated from milk samples obtained in four dairy herds which were experiencing considerable trouble with mastitis.

unit of bacitracin diluted from 512 to 1,024 times produced bacteriostasis of groups A, B, C, and G streptococci. On the other hand, the six strains of *Str. uberis* and 20 strains of hemolytic staphylococci proved to be susceptible to the action of bacitracin, but considerable difference in sensitivity was found to exist between different strains of organisms in these groups.

In view of the comparatively greater resistance of *Str. agalactiæ* to bacitracin and

TABLE 5—Results of Treating Infected Udder Quarters with Bacitracin

Cow	Quarter	Type of Infection	Treatment			Results of treatment
			1st dose	Interval	2nd dose	
1	R.F.	*Str. agalactiae*	41,000 u.	.....	.....	Infection not eliminated
2	L.F.	*Str. agalactiae*	41,000 u.	24 hr.	41,000 u.	Infection not eliminated
3	L.R.	*Str. uberis*	41,000 u.	24 hr.	41,000 u.	Infection not eliminated
4	R.F.	*Str. dysgalactiae*	41,000 u.	24 hr.	41,000 u.	Infection not eliminated
5	L.F.	*Str. agalactiae*	123,000 u.	.....	.....	Infection not eliminated
	R.R.	*Str. agalactiae*	123,000 u.	.....	.....	Infection not eliminated
	L.R.	*Str. agalactiae*	123,000 u.	.....	.....	Infection not eliminated
6	L.F.	*Str. agalactiae*	164,000 u.	.....	.....	Infection not eliminated
	L.R.	*Str. agalactiae*	164,000 u.	.....	.....	Infection not eliminated

R.F. = right front quarter; L.F. = left front quarter; R.R. = right rear quarter; L.R. = left rear quarter.

All the cultures gave typical biochemical reactions for their respective types, with the exception of a part of the strains of *Streptococcus agalactiæ*, used in the work with bacitracin, which failed to ferment trehalose.

As shown in table 1, all the organisms tested were sensitive to the action of subtilin. However, considerable difference in sensitivity was found to exist between different strains of the same group of organisms. Of the 33 cultures of *Str. agalactiæ* tested, 30 cultures were completely inhibited by 11.9 μg. of subtilin per cubic centimeter of medium, while two cultures grew slightly at this concentration, and one culture gave slight growth at the next higher concentration of 23.8 μg. The ten cultures of *Streptococcus uberis* were of the same order of sensitivity to the action of subtilin, being completely inhibited by a concentration of 2.9 μg. per cubic centimeter of medium. The 33 strains of hemolytic staphylococci tested were completely inhibited by 23.8 μg. of subtilin per cubic centimeter of medium, while ten of the strains were inhibited by a concentration as low as 1.4 μg. One strain of hemolytic staphylococci, which, in previous tests, was found to be resistant to the action of penicillin, proved to have the same sensitivity to subtilin as the average for other strains of this organism tested.

As indicated in table 2, the 31 cultures of *Str. agalactiæ* tested were all resistant to a concentration of two units of bacitracin per cubic centimeter of medium. These results are in contrast to results of tests reported by Johnson and coworkers,[3] who found that one

the fact that a significant synergistic action between penicillin and bacitracin had been demonstrated in the treatment of experimental syphilis,[6] it was decided to test the action of a combination of these two antibiotics against mastitis streptococci and staphylococci *in vitro*.

A comparison of results tabulated in table 2 and table 3 indicates that, by combining bacitracin and penicillin in the test medium, the concentration of bacitracin necessary to inhibit the growth of *Str. agalactiæ* and *Str. uberis* was reduced by at least four multiple dilutions and, in the case of hemolytic staphylococci, by three dilutions. At the same time, a comparison of these results with the results obtained in similar work with penicillin[5] shows that the concentration of penicillin necessary to inhibit the growth of *Str. agalactiæ* was reduced by approximately one half when combined with bacitracin. The reduction in the concentration of penicillin necessary to inhibit the growth of the other organisms tested was even more marked, being approximately twofold in the case of *Str. uberis* and hemolytic staphylococci. As a result of this work, it is believed that a definite synergistic action has been demonstrated between penicillin and bacitracin for streptococci and hemolytic staphylococci commonly associated with bovine mastitis.

MATERIALS AND METHODS

A majority of the cows used in these experiments were a part of a small experimental

herd maintained for research on bovine mastitis at the Bureau of Animal Industry's Animal Disease Station, Beltsville, Md. These cows were housed and otherwise cared for under conditions comparable to those found in the average commercial herd. A part of the cows had been artificially infected with streptococci or staphylococci, whereas some of the cows had contracted the infection naturally. The course of udder infections and changes in the milk of the cows in this herd were followed by obtaining quarter milk samples from each lactating cow each week and submitting them to bacteriologic and chemical tests. In the case of dry cows, diagnosis of infection was based on a bacteriologic examination of a sample of secretion from the nonlactating gland obtained prior to treatment, and the results of treatment were determined by a bacteriologic examination of a sample of secretion obtained at a suitable interval after treatment or an examination of the colostrum. When cows outside of the experimental herd were used, the presence of infection was determined by two bacteriologic examinations of milk samples obtained from each cow prior to treatment and an examination of milk samples obtained at least two weeks following treatment.

All treatments were administered by udder infusion. Unless otherwise indicated, a standard procedure was followed by dissolving the material to be infused into each quarter in 50 cc. of sterile distilled water and employing a 50-cc. glass syringe equipped with a blunt-pointed cannula to administer the infusion.

Subtilin.—When the clinical work with subtilin was started there was no information available regarding the effect of this antibiotic on mammary tissue. Preliminary trials with infusions into normal, noninfected udders indicated that subtilin was well tolerated by mammary tissue.

As shown in table 4, treatment with subtilin caused elimination of the infection from only two of 15 *Str. agalactiæ*-infected quarters and did not have any permanent effect on the infection in three *Streptococcus dysgalactiæ*-infected quarters or on the infection in four hemolytic Staphylococcus-infected quarters. However, there was a temporary reduction of bacteria in the milk and a temporary improvement in the clinical condition of the udder and physical appearance of the milk following treatment.

Bacitracin.—As in the case of subtilin, preliminary work indicated bacitracin was well tolerated by udder tissue and caused no noticeable disturbance of milk production.

The results of treatment with bacitracin are given in table 5. It will be noted that from the standpoint of bacteriologic cures, bacitracin was ineffective in the dosage employed in this experiment. However, as in the case of subtilin, there was in most cases a marked temporary improvement in the clinical condition of the udder and physical appearance of the milk following treatment.

Because of the lack of permanent benefit resulting from the use of bacitracin alone and in view of the fact that a definite synergistic action between bacitracin and penicillin was found when tested against mastitis streptococci and staphylococci *in vitro*, it was decided to test a combination of these two agents clinically.

As shown in table 6, the results obtained from treating various types of udder infec-

TABLE 6—Results of Treating Infected Udder Quarters with a Combination of Bacitracin and Penicillin

Cow	Quarter	Type of infection	Treatment			Results of treatment
			1st dose	Interval	2nd dose	
1	R.F.	*Str. uberis*	41,000 u.bac. 50,000 u.pen.	.....	.....	Infection not eliminated
	L.F.	*Str. uberis*	50,000 u.pen.	.....	.....	Infection not eliminated
2	R.F.	*Str. dysgalactiae*	41,000 u.bac. 50,000 u.pen.	.....	.....	Infection not eliminated
x2	R.F.	*Str. agalactiae*	50,000 u.pen.	.....	.....	Infection not eliminated
	R.R.	*Str. agalactiae*	50,000 u.pen.	.....	.....	Infection not eliminated
	L.F.	Staph.	50,000 u.pen.	.....	.....	Infection not eliminated
	L.F.	Staph.	50,000 u.pen.	.....	.....	Infection not eliminated
4	R.R.	*Str. uberis*	41,000 u.bac. 50,000 u.pen.	24 hr.	41,000 u.bac. 50,000 u.pen.	Infection not eliminated
5	R.F.	Staph.	50,000 u.pen.	24 hr.	50,000 u.pen.	Infection not eliminated
	L.F.	Staph.	50,000 u.pen.	24 hr.	50,000 u.pen.	Infection not eliminated
	R.R.	Staph.	50,000 u.pen.	24 hr.	50,000 u.pen.	Infection not eliminated
6	R.F.	Staph.	50,000 u.pen.	24 hr.	50,000 u.pen.	Infection eliminated
	L.F.	Staph.	50,000 u.pen.	24 hr.	50,000 u.pen.	Infection not eliminated
7	R.F.	Staph.	50,000 u.pen.	24 hr.	50,000 u.pen.	Infection not eliminated
8	L.R.	Staph.	50,000 u.pen.	24 hr.	50,000 u.pen.	Infection not eliminated

x = dry cow; R.F. = right front quarter; L.F. = left front quarter; R.R. = right rear quarter; L.R. = left rear quarter; Staph. = hemolytic staphylococci.

tions with a combination of bacitracin and penicillin were not significantly better than with bacitracin alone.

DISCUSSION

In view of the demonstrated susceptibility of mastitis streptococci and staphylococci to subtilin and to a combination of bacitracin and penicillin *in vitro*, and the conclusion by Benson[7, 8] that bacitracin in dosages of 20,000 to 100,000 units per udder quarter shows definite promise as a therapeutic agent for the treatment of *Str. agalactiæ* mastitis, the results of the clinical trials contained in this report were disappointing. It is possible that a larger number of infusions with either agent, as Benson[8] employed in his work with bacitracin, would have given a higher percentage of cures. Other factors which undoubtedly operated to make this a rather rigorous test were that a majority of the cows were showing some physical evidence of mastitis either in the form of abnormal milk or varying degrees of udder induration. However, as stated before, the purpose of this work was to find an agent or method of treatment which would not only be equal to but superior in at least some respects to the agents of proved value, such as penicillin or sulfanilamide, which are generally available for the treatment of this disease. In the light of the results obtained thus far, it is not considered that either subtilin or bacitracin has given evidence of being a superior agent for the treatment of bovine mastitis.

SUMMARY

The results of *in vitro* and *in vivo* tests of subtilin, bacitracin, and a combination of bacitracin and penicillin as possible treatments for mastitis are described.

In sensitivity tests, both subtilin and bacitracin in combination with penicillin proved to be effective against microörganisms commonly associated with bovine mastitis.

Clinical trials were disappointing in that neither subtilin nor bacitracin proved to be superior to agents now available for treating this disease.

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Studies on the Efficacy of Aureomycin and Aureomycin-Sulfadiazine in Experimental Brucellosis in Guinea Pigs

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A COMBINATION of streptomycin and sulfadiazine has been found to produce satisfactory cures in a high proportion of cases of human brucellosis[7] and experimental brucellosis in guinea pigs.[3] Furthermore, it has been shown in experimental infections of guinea pigs that the combination was as effective when given every other day as when given every day even though therapy was started ten days after infection.[4]

Whereas dihydrostreptomycin may replace streptomycin in the sulfonamide combination, toxicity caused by the sulfonamides themselves is common during the course of treatment in human patients. This toxicity has not been observed in our work with guinea pigs. Reports from several sources[2,6,7] indicated that the toxicity problem in human therapy may be circumvented by the use of aureomycin as an anti-Brucella agent.

Spink *et al.*[6] reported on the effect of oral aureomycin on *Brucella melitensis* infections in Mexico. Latest information indicated that only 3 of the 24 treated patients showed recurrence of fever and positive blood cultures.

Heilman[1] combined aureomycin and dihydrostreptomycin in treating experimental Brucella infections in mice and concluded that this combination was superior to streptomycin-sulfadiazine and aureomycin-sulfadiazine. However, under the conditions of the experiment, no combination completely eliminated the organisms from the spleen.

Using Heilman's approach, Herrell[2] employed aureomycin-dihydrostreptomycin in treating 4 clinical cases of brucellosis. Three of the cases were caused by *Brucella abortus* and 1 by *Brucella suis*. Good responses were obtained in all cases. No relapses were reported.

In the reports cited above, no serious toxicity was observed. The aureomycin was administered orally in all cases. Some nausea, vomiting, and diarrhea were observed, and Spink reported that in 50 per cent of the cases given 500 mg. every six hours that a spike in the temperature occurred with occasional drop in blood pressure and tachycardia.

Lederle Laboratories[5] reported that, of the animals tested, guinea pigs did not tolerate the antibiotic well. Administration of 40 mg. per kilogram for eight days may result in death. Because the anticipated dosage of the drug was considerably lower than this figure, guinea pigs were again used in the studies reported here. The use of guinea pigs was considered advisable in our work to make a more direct comparison between the efficacies of streptomycin-sulfadiazine and aureomycin in treating comparable diseases due to the same virulent bovine strain of Brucella.

METHODS

Male and female guinea pigs of 400 to 500 Gm. body weight were used. Each animal was inoculated intraperitoneally with approximately 1 million cells of a 24-hour tryptose broth culture of *Br. abortus* strain 2308.

The animals were grouped into lots of 10 and treated according to the following schedule. A second trial was run after completion of the first. Each group comprised 20 animals.

1) Controls—infected; no treatment.
2) Treatment initiated at the time of infection.
   a) Four treatments per day.
      1—Aureomycin 1.25 mg. at 8 a.m., 11 a.m., 2 p.m., and 5 p.m.
      2—Aureomycin 1.25 mg. plus 30 mg. sulfadiazine at 8 a.m., 11 a.m., 2 p.m., and 5 p.m.
   b) Two treatments per day.
      1—Aureomycin 2.50 mg. at 8 a.m. and 5 p.m.

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At present, Dr. Holm is at the School of Veterinary Medicine, University of California, Davis.

2—Aureomycin 1.25 mg. at 8 a.m. and 5 p.m.
3—Aureomycin 1.25 mg. plus 60 mg. sulfadiazine at 8 a.m. and 5 p.m.

3) Treatment initiated ten days after infection.
a) Four treatments per day.
1—Aureomycin 1.25 mg. at 8 a.m., 11 a.m., 2 p.m., and 5 p.m
2—Aureomycin 1.25 mg. plus 30 mg. sulfadiazine at 8 a.m., 11 a.m., 2 p.m., and 5 p.m.
b) One treatment per day.
1—Aureomycin 5 mg. at 8 a. m.
2—Aureomycin 5 mg. plus 120 mg. sulfadiazine at 8 a.m.

Aureomycin, as the hydrochloride, was administered subcutaneously in 0.5-ml. doses. Sodium sulfadiazine was administered in 0.5-ml. oral doses. Although subcutaneous, intraperitoneal, and intramuscular injections of the hydrochloride of aureomycin were known to be somewhat irritant, the amount of the antibiotic available at the time of the trials was such that the subcutaneous route of administration was utilized to conserve the supply.

Treatment was continued for twenty-one days and therapeutic efficacy was determined as in previous trials.

RESULTS

The results are shown in table 1. Subcutaneous injection of aureomycin hydrochloride produced slight local reaction at the injection site, and the reaction did not appear to be serious in these instances.

At the dosage level used (maximum of 12.5 mg./kg.), the animals lost weight rapidly, reaching a peak of weight loss among the survivors of 10 to 50 Gm. within the first week of treatment. The greatest losses occurred among animals receiving the aureomycin-sulfonamide combination. Those animals which eventually died became so emaciated that they could not stand or move about. Gross findings at autopsy showed a complete loss of body fat. There appeared to be no correlation between the number of deaths in a group and the frequency of or the size of the dose. There was a tendency for a greater number of deaths to occur in those groups receiving both aureomycin and sulfadiazine.

Treatment started ten days after infection produced the best results, four treatments per day being somewhat superior to a single treatment per day, and the aureomycin-sulfonamide combination being somewhat superior to aureomycin alone.

These data confirmed those of Heilman in indicating that the combination was not completely effective in eliminating Brucella

TABLE 1—Efficacy of Aureomycin and Aureomycin-Sulfadiazine in the Treatment of Brucellosis in Guinea Pigs

Treatment	Deaths	Lesions of survivors: Severity	Lesions of survivors: Per cent with	Agglutination test titers of survivors: Average Initial	Average Final	Per cent with Initial	Per cent with Final
1) Controls	0	4+	100	1 : 400	1 : 400	100	100
2) Treatment initiated at time of infection							
a) Four treatments per day							
1—Aureomycin	5/20	2+	80.0	1 : 25	1 : 200	60	67
2—Aureomycin-sulfadiazine	7/20	2+	77.0	1 : 25	1 : 200	31	62
b) Two treatments per day							
1—Aureomycin at 2.50 mg. x 2	4/20	2+	75.0	1 : 25	1 : 100	50	100
2—Aureomycin at 1.25 mg. x 2	2/20	2+	100	1 : 200	1 : 200	100	78
3—Aureomycin at 1.25 mg. plus sulfadiazine 60 mg. x 2	9/20	2+	54.5	1 : 25	0	82	0
3) Treatment initiated ten days after infection							
a) Four treatments per day							
1—Aureomycin	4/20	1+	44.0	1 : 100	1 : 25	62	31
2—Aureomycin-sulfadiazine	6/20	0	0	1 : 50	0	71	0
b) One treatment per day							
1—Aureomycin	2/20	1+	50	1 : 25	1 : 100	11	67
2—Aureomycin-sulfadiazine	6/20	1+	14	1 : 50	1 : 100	50	29

from the spleens of infected animals. In this respect, under comparable conditions, the streptomycin-sulfadiazine combination proved to be more effective.

DISCUSSION

Although aureomycin-sulfadiazine did not appear to be as effective as streptomycin-sulfadiazine when given together in guinea-pig brucellosis, it may not follow that the former would be less effective or that either combination would be effective in treating brucellosis in cattle or swine. Extrapolation of results from guinea pigs to cattle or swine may be as hazardous as extrapolation of data from *in vitro* to *in vivo* conditions. Spink[6] reported that aureomycin was not as effective as streptomycin in *in vitro* tests or in tests with Brucella-infected chicken embryos.

Available toxicity data indicated that aureomycin was not markedly toxic to cows. Lederle Laboratories[5] stated that an initial intravenous injection of 5 mg. per pound plus seven maintenance injections of 2.5 mg. per pound at twelve-hour intervals did not provoke any untoward physical signs. No data are available for swine.

SUMMARY

Aureomycin and sulfadiazine given simultaneously by the subcutaneous and oral routes, respectively, proved to be effective in reducing spleen size in experimental brucellosis in guinea pigs, but they were not as effective as streptomycin and sulfadiazine in eliminating the infection. Treatment begun ten days after infection produced the best results. Administration of aureomycin and aureomycin plus sulfadiazine produced toxic effects in guinea pigs, the combination being more toxic than aureomycin alone.

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Studies on the Effect of Streptomycin in Experimental Brucellosis in Guinea Pigs

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THERE is a growing tendency among investigators in the field of antibiotic therapeutics to consider the value of intermittent treatment. From a practical standpoint, such treatment has much to offer since constant blood and tissue levels of the antibiotic may be difficult to maintain under field conditions. Although Spink *et al.*[6] reported that *Brucella abortus* strains isolated from 3 patients given streptomycin and sulfadiazine showed no increase in resistance to streptomycin, it is possible that resistant variants could appear when the Brucella population is subjected to constant drug concentrations. No data are at hand, but it could seem logical that intermittent treatment would reduce the chance of the appearance of resistant variants.

The studies here reported[a] have been extended to compare the efficacy of streptomycin-sulfadiazine when administered once daily with the efficacy of the combination when administered on alternate days, the total dosage being the same in each case.

METHODS

Male and female, 400- to 500-Gm guinea pigs were used in this series of trials. Each animal was inoculated intraperitoneally with approximately 1 million cells of a 24-hour tryptose broth culture of *Br. abortus* strain 2308.

The animals, grouped into lots of 10, were treated according to the following schedule:

1) Controls—infected; no treatment.

2) Treatment initiated at the time of infection.
   a) One treatment per day.
      Group 1—10,000 units of streptomycin per day.
      Group 2—120 mg. of sodium sulfadiazine per day.
      Group 3—10,000 units of streptomycin plus 120 mg. of sodium sulfadiazine.
   b) One treatment on alternate days.
      Group 1—20,000 units of streptomycin every other day.
      Group 2—240 mg. of sodium sulfadiazine every other day.
      Group 3—20,000 units of streptomycin and 240 mg. of sodium sulfadiazine every other day.
      Group 4—20,000 units of streptomycin and 120 mg. of sodium sulfadiazine every other day.

3) Treatment initiated ten days after infection.
   a) One treatment per day.
      Groups 1, 2, and 3 same as in 2*a*.
   b) One treatment every other day.
      Groups 1, 2, 3, and 4 same as in 2*b*.

In each instance, the $CaCl_2$ complex of streptomycin was administered subcutaneously in 0.5-ml. doses. Sulfadiazine was administered, as the sodium salt, in a 0.5-ml. oral dose.

As in the previous trial, treatment was continued for twenty-one days. On the twenty-second day, the animals were bled for the determination of Brucella agglutination titers. Twenty days later, the animals were sacrificed, blood collected, and examination made for pathologic changes. Comparable samples of the liver, spleen, lymph nodes, and, in the case of male animals, the epididymis were macerated and smeared on tryptose agar plates. The plates were checked after six days' incubation for the presence of Brucella colonies, and the relative numbers of the colonies were recorded.

RESULTS

The results of the experiment are shown in table 1. The criteria for lesions and titers were the same as those reported by Holm and McNutt[1] in previous trials; *i. e.*, lesions represented gross changes in the organs, especially in the spleen, and confirmation of these by the density of Brucella colonies on the agar plates. As in the previous trial, there was good correlation between the gross lesions and the numbers of colonies isolated on the plates.

Initial titers represent the average titers determined at the time treatment ceased.

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[a] Since the inception of this work, aureomycin has been reported to show promise as a therapeutic agent for human brucellosis. Heilman[2] reported that in mice, aureomycin combined with dihydrostreptomycin was more effective in reducing the number of Brucella in the spleen than was dihydrostreptomycin and a sulfonamide. Our observations on the use of aureomycin for guinea-pig brucellosis will appear separately.

Final titers were those obtained at autopsy twenty days later. The figures for the average titer represent the average for all animals in a group, even though some may have had no titers.

All controls were severely infected; the spleens and lymph nodes were greatly enlarged. Lesions of the epididymis were numerous. Titers were high and Brucella organisms were recovered in large numbers.

On all schedules, the combination of streptomycin and sulfadiazine proved to be markedly better than either alone. The results obtained on one treatment each day starting at zero time or at ten days after inoculation confirmed earlier results obtained in this laboratory.

Treatment on alternate days produced as high a percentage of tissue sterilizations as that given on every day. It is significant that results obtained with 20,000 units of streptomycin and 120 mg. of sulfadiazine were as good as those obtained when 240 mg. of sulfadiazine was used in the combination.

DISCUSSION

In the experience of Eisele, reported by Spink,[6] the combination of streptomycin and sulfadiazine was moderately toxic when given to human subjects at a level of 2 Gm. of streptomycin per day. Toxicity was also mentioned by Herrell.[3] In our experience, the combination has not proved to be toxic for guinea pigs. The dosage used in our trials was about three-fourths that used by Eisele. However, the administration of the combination to cows at a level of 1 Gm. of streptomycin per 100 lb. of body weight per day for ten days has resulted in some vestibular dysfunction.[7] It is possible that the toxicity in human subjects and in cows might be circumvented by the use of dihydrostreptomycin.

No investigations have been made on the resistance of isolated organisms to streptomycin in this study. In previous trials with around-the-clock injection schedules, organisms isolated from streptomycin-treated animals were resistant to 100 times the units necessary to inhibit the parent cul-

TABLE 1—Comparative Efficiencies of Streptomycin, Sulfadiazine, and Combinations of Streptomycin-Sulfadiazine when Given to Brucella-Infected Guinea Pigs Once per Day and Once Every Other Day

	Lesions		Agglutination test titers			
		No. positive	Average		Per cent with	
	Severity	No. treated	Initial	Final	Initial	Final
1) Controls	4+	10/10	1 : 400	1 : 400	100	100
2) Treatment at the time of infection						
a) One treatment per day						
1—Streptomycin	2+	10/10	1 : 125	1 : 125	100	100
2—Sulfadiazine	2+	8/10	1 : 50	1 : 50	30	70
3—Streptomycin-sulfadiazine	1+	3/10	0	1 : 50	0	30
b) One treatment every other day						
1—Streptomycin	3+	9/10	1 : 150	1 : 250	100	90
2—Sulfadiazine	3+	10/10	1 : 100	1 : 100	90	100
3—Streptomycin plus 120 mg. of sulfadiazine	0	0/10	0	1 : 25	0	10
4—Streptomycin plus 240 mg. of sulfadiazine	1+	3/10	0	1 : 25	0	10
3) Treatment started ten days after infection						
a) One treatment per day						
1—Streptomycin	3+	9/10	1 : 300	1 : 300	100	100
2—Sulfadiazine	2+	10/10	1 : 175	1 : 75	100	100
3—Streptomycin-sulfadiazine	1+	3/10	1 : 100	1 : 25	60	10
b) One treatment every other day						
1—Streptomycin	2+	8/10	1 : 200	1 : 250	100	80
2—Sulfadiazine	2+	6/10	1 : 150	1 : 125	50	80
3—Streptomycin plus 120 mg. of sulfadiazine	1+	1/10	1 : 50	1 : 25	60	10
4—Streptomycin plus 240 mg. of sulfadiazine	1+	1/10	1 : 50	1 : 25	50	10

ture. Hall and Spink[1] reported that *Br. abortus* in the blood of a patient with subacute bacterial endocarditis due to Brucella was extremely resistant to streptomycin *in vitro* after the patient had received a one month's course of streptomycin therapy. Spink *et al.*[6] found that streptomycin-resistant strains did not appear after combined therapy.

SUMMARY

Subcutaneous administration of streptomycin plus oral administration of sulfadiazine on alternate days was as effective as daily treatments, the total dosage of the agents having been the same in each instance. Treatment every other day was as effective when it was started at the time of infection as when ten days were allowed to elapse before treatment was started. Under the conditions of the experiment, *Brucella abortus* was not isolated from the spleens of 70 to 100 per cent of the treated animals.

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The Deleterious Effects of Flat Pea Seed for Rats

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THE FLAT PEA (*Lathyrus silvestris wagneri*) is a long-lived perennial legume. Although it is difficult to obtain satisfactory stands, it is a valuable cover crop for cut-over timber land and other areas unsuitable for cultivation in western Washington and Oregon. Flat peas yield a large amount of high quality feed, both forage and seeds being palatable to sheep and cattle. Occasional reports, however, have been made concerning the toxicity of flat peas to sheep or laboratory animals when fed either as forage or seed. Little work has been done on this toxicity. The work herein reported was undertaken in order to study the deleterious effects of flat peas for rats and to characterize the general nature of the toxic agent present. Inasmuch as many of the symptoms of "flat pea poisoning" resemble those observed in deficiencies of certain of the B vitamins, an attempt was made to determine if the toxic factor was antivitamin in nature.

Hodgson[1] and Daniel[2] reported the death of sheep fed flat peas. Lewis[3] likewise found that both flat pea hay and seed were toxic to a high degree for rats. He found this toxicity was reduced by extraction with ethyl alcohol (30%) but not with ether. Miller[4] reported that 20 per cent of flat peas used as protein supplement for day-old chicks resulted in the death of 40 per cent of the birds. In 1946, Miller[5] reported that, while a large, single dose of flat pea seed (100,000 mg./kg. body weight) did not kill 11-day-old chicks, the continued feeding of the seed at a 50 per cent level resulted in death to all 15-day-old birds. Haag[6] observed that flat peas were toxic to guinea pigs.

TABLE I—Effect of Vitamin Supplementation upon the Toxicity of Flat Peas for Weanling Rats

No. rats	Ave. init. wt.	Ration	Vitamin supplement	Deaths						Total
				6 da.	7 da.	8 da.	9 da.	11 da.	14 da.	
11	48 Gm.	Basal	None							0
11	48 Gm.	Basal + 20% flat peas	None	7	1		1			9
5	48 Gm.	Basal + 40% flat peas	None	5						5
5	48 Gm.	Basal + 20% flat peas	1 mg./100 Gm. ribo. in ration	4				1		5
5	48 Gm.	Basal + 40% flat peas	1 mg./100 Gm. ribo. in ration	5						5
5	48 Gm.	Basal + 20% flat peas	10 B vitamins[1] in ration	4	1					5
5	48 Gm.	Basal + 40% flat peas	10 B vitamins[1] in ration	4	1					5
6	48 Gm.	Basal + 20% flat peas	10 B vitamins[2] in ration	2			1			3
6	48 Gm.	Basal + 20% flat peas	10 mg./100 Gm. pyridoxine in ration	2	3	1				6
6	48 Gm.	Basal + 20% flat peas	10 mg./100, 2 mg./100 ribo. + thiamine in ration	1			1			2
6	48 Gm.	Basal + 20% flat peas	15% alfalfa	5						5

[1]Thiamine, 2 mg.; riboflavin, 10 mg.; choline, 1 Gm.; pyridoxine, 2 mg.; pantothenic acid, 6 mg.; niacin, 2.5 mg.; inositol, 3 Gm.; para-aminobenzoic acid, 300 mg.; folic acid, 2 mg.; and biotin, 0.5 mg. per kilogram ration.

[2]Thiamine, 20 mg.; riboflavin, 100 mg.; choline, 10 Gm.; pyridoxine, 20 mg.; pantothenic acid, 60 mg.; niacin, 25 mg.; inositol, 30 Gm.; para-aminobenzoic acid, 3 Gm.; folic acid, 20 mg.; and biotin, 5 mg. per kilogram of ration.

EXPERIMENTAL

A series of experiments were conducted using Sprague-Dawley rats, varying in age from weanlings to adults.

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The following basal ration was used in all experiments: wheat, 12.5 per cent; barley, 12.0 per cent; cull Alaska peas, 69.0 per cent; alfalfa, 5.0 per cent; salt, 0.5 per cent; oyster shell, 0.5 per cent; and steamed bone meal, 0.5 per cent. Because the flat pea seed used in the experiments* contained approximately 25 per cent protein (the same percentage as was contained in the cull Alaska peas), the flat pea seed incorporated in the ration was substituted for an equal amount of the latter ingredient without appreciable change in the protein content of the ration.

The flat pea seed used was finely ground and mixed directly into the ration.

A modified paired-feeding technique was used whereby the daily amount of ration for each rat was regulated by the smallest amount consumed by any 1 animal on the previous day. The length of each experiment varied with the survival of the animals. All rats were weighed weekly.

Trials were designed to learn the following facts concerning the toxicity of flat pea seed: (1) the effect of supplementation with B-complex vitamins at both high and low levels; (2) the relationship between age and weight and tolerance to flat pea toxin; and (3) the stability of the toxic principle in flat peas to heat treatment.

RESULTS

General Syndrome of Flat Pea Poisoning.—The effects of the toxin found in flat pea seed on rats were similar in all cases. Symptoms began with anorexia, followed by a very rapid decline in weight. During this period, there appeared a hypersensitivity to external stimuli. This hypersensitive state progressed into convulsions which increased in frequency and severity. During this time, excessive salivation and licking of the forepaws was observed. Convulsions were followed by periods of extreme exhaustion, increasing in length until the rats fell into a deep coma. Coma was followed by death. The toxicity symptoms were found to be irreversible in cases where the flat peas were withdrawn from the ration after their appearance.

Several of the adult rats which had died of the flat pea toxin were autopsied. The only macroscopic lesions observed in these animals were in the intestinal tract. Pronounced distention of the intestines and stomach was found. In most cases, the walls of the tract were paper thin and practically transparent. A hard, compact mass was found in the large intestines of 2 of the dissected rats. The lesions described above were found only in animals which had received the flat pea seed over extended periods. Kidney hemorrhage and bone malformation, as were observed in chicks by Miller,[6] were not evident.

Effect of Supplemental B Vitamins and Alfalfa.—The mortality data for these experiments are summarized in tables 1 and 2.

TABLE 2—Deaths Resulting at Increasing Levels of Flat Peas in Ration of 85 Gm. Rats

No. rats	Ave. init. wt.	Vitamin supplement	Flat peas in ration						Total deaths
			10% 3 wk.	20% 2 wk.	30% 3 wk.	35% 2 wk.	40% 2 wk.	45% 2 wk.	
6	85 Gm.	None							0
6	85 Gm.	None		1*	2		1	2	6
4	85 Gm.	250 ribo. injected daily			1		1	2	4
4	85 Gm.	200 ribo. injected daily			2		2		4
6	85 Gm.	Ribo. added to ration (10 mg./100 Gm. feed)			5		1		6
6	85 Gm.	10 B vitamins to ration. High level			1		5		6

*This death was evidently not due to flat pea toxicity, inasmuch as it exhibited none of the toxicity symptoms.

Vitamins fed at 60 times the normal requirements or thiamine, 20 mg.; riboflavin, 100 mg.; choline, 10 Gm.; pyridoxine, 20 mg.; calcium pantothenate, 60 mg.; niacin, 25 mg.; inositol, 20 Gm.; para-aminobenzoic acid, 3 Gm.; folic acid, 20 mg.; and biotin, 5 mg. per kilogram of feed.

Table 1 shows the effect of high and low levels of thiamine, riboflavin, and a combination of all ten of the known B-complex vitamins with rations containing 20 and 40 per cent flat peas. The rats used in these experiments were weanlings, with an average initial weight of 48 Gm. As can be seen, a level of 20 per cent flat peas was highly toxic to rats of this age. Of a total of 45 animals given 20 per cent flat peas, 25 were dead from the toxin on the seventh day, regardless of treatment. By the fourteenth day, 35 of the 45 had died. The feeding of low levels of the B-complex vitamins

*The flat pea seed used in these experiments was supplied by Dr. Newell Dixon, grazing specialist of the Western Washington Experiment Station, Puyallup.

or of 15 per cent alfalfa had no apparent effect upon the mortality due to flat pea toxin. When a level of 40 per cent of flat peas was given, the mortality was even higher. All rats fed this level, regardless of supplement, were dead at the end of seven days. There were indications that high levels of the ten B vitamins (60 times normal requirement) had an effect upon the length of life of the rats receiving 20 per cent flat peas. Lower levels of these vitamins, however, as well as a 15 per cent level of alfalfa in the ration, gave no apparent beneficial results.

TABLE 3—Weekly Gains in Grams of 85-Gm. Rats Fed Low Levels of Flat Peas During Six Weeks' Feeding Period

Flat pea percentage	Weeks	Lot 1	2	3	4	5	6
10	Initial	83	86	89	88	83	83
10	First	72	82	90	82	83	83
10	Second	88	99	103	92	96	102
10	Third	91	106	109	106	103	107
20	Fourth	116	120	130	120	113	124
20	Fifth	123	134	145	132	123	130
20	Sixth	138	139	151	138	137	141

Table 2 shows the results of a trial in which the level of flat peas was increased by steps over a period of fifteen weeks. The rats used in these experiments averaged 85 Gm. in weight. As in the previous trials, lots were supplemented with riboflavin at a level of 250 μg. per 100 Gm. of ration and also with the ten B-complex vitamins at levels 30 and 60 times the accepted normal requirements. In addition, two lots were given daily injections of 250 μg. of riboflavin or 200 μg. of thiamine. In contrast to the weanling rats, the older rats used in these trials tolerated 10 and 20 per cent of the flat peas. In fact, as can be seen in the gain data of table 3, covering the first six weeks of the experiment, their gains were at least equal to those of the control group containing no flat peas.

Symptoms of toxicity began to appear during the seventh week, when the level of flat peas had been raised to 30 per cent. Of 26 rats, 12 were dead at the end of the ninth week. There were no survivors at the end of fourteen weeks, at which time a level of 45 per cent flat peas was being fed in the ration. The vitamin supplements given had no detectable value in overcoming the toxic principle of flat pea seeds.

There was no apparent value in the administration of large therapeutic doses of thiamine and riboflavin, either separately or in combination, to rats which exhibited the symptoms of flat pea toxicity. Likewise, treatment by the injection of 250 μg. of pyridoxine or 50 mg. of ascorbic acid had no effect in alleviating convulsions brought about by the ingestion of flat pea seed. Methionine mixed with the ration containing flat peas, likewise, showed no beneficial results.

Effect of Age.—Further study was made of the indicated relationship between age, weight, and tolerance to the flat pea toxin. The tolerance to peas, as measured by survival time, was determined for rats weighing 150, 200, and 250 Gm., to levels of 30, 40, 50, and 60 per cent of flat pea seed in the rations. These data are summarized in table 4. It can be seen that all rats of this size were able to tolerate 30 per cent of the peas. They were all comparable with the controls, both in weight and appearance, at the end of 105 days. None of the rats, however, tolerated the 40 per cent level. Although any test for significance was impossible, the average number of days of survival indicated that the 250-Gm. rats were able to survive longer than those weighing 150 to 200 Gm. This trend is most evident at the higher levels of 50 and 60 per cent flat peas.

TABLE 4—Effect of Age and Weight upon Susceptibility to Flat Pea Toxicity

No.	Ration	Ave. init. wt.	Days survival	Ave. days survival
2	Basal	150	105, 105	105*
2	Basal	200	105, 105	105*
2	Basal	250	105, 105	105*
2	Basal + 30% flat peas	150	105, 105	105*
2	Basal + 30% flat peas	200	105, 105	105*
2	Basal + 30% flat peas	250	105, 105	105*
2	Basal + 40% flat peas	150	21, 34	28
2	Basal + 40% flat peas	200	18, 8	13
2	Basal + 40% flat peas	250	34, 72	53
2	Basal + 50% flat peas	150	11, 7	9
2	Basal + 50% flat peas	200	8, 15	12
2	Basal + 50% flat peas	250	17, 9	13
2	Basal + 60% flat peas	150	9, 4	7
2	Basal + 60% flat peas	200	11, 11	11
2	Basal + 60% flat peas	250	17, 16	17

*At the end of this period, all rats of this group appeared normal.

Heat Stability of Flat Pea Poison.—In order to determine the stability of the toxic factor to heat, quantities of flat pea seed were autoclaved with and without added moisture at a pressure of 15 lb. (120 C.)

for thirty minutes. These treated flat peas were incorporated in the basal ration at a level of 60 per cent and fed to adult rats. All animals receiving the flat peas (autoclaved either with or without added water) in this trial died with the typical symptoms of the poisoning within three days after the initiation of the trial.

SUMMARY

It can be concluded from the data obtained that vitamin supplementation with all ten known members of the B-complex vitamins, even at levels which were 60 times the normal requirement, had no appreciable effect upon tolerance of rats to the toxic principle found in flat pea seed. Although there was inconclusive evidence that, in the case of small weanling rats, the survival time was lengthened by the addition of high levels of the B vitamins; in all cases, the rats eventually exhibited the typical symptoms of the poison. Injections of large doses of thiamine, riboflavin, pyridoxine, or ascorbic acid had no effect in alleviating the toxicity syndrome. Methionine mixed in the ration, likewise gave no beneficial results. The toxic substance is evidently not an antivitamin, but we believe that it may be alkaloid in nature. The toxic principle can be extracted from the seed by ethyl alcohol (30%) and is stable to drastic heat treatment.

Data obtained indicate that there is a definite relationship between age and increased body weight and tolerance by rats to the toxic substance. Small weanling rats were unable to survive the feeding of 20 per cent of the flat peas in the diet, while rats weighing 85 Gm. and 200 to 250 Gm. were able to tolerate 20 and 30 per cent, respectively. The actual limit of flat pea level for adult rats appears to lie between 30 and 40 per cent. There is some evidence that adult rats, at least, may be able to build up a tolerance to the poison when it is given over a prolonged period, inasmuch as a few rats survived until a 45 per cent level of the flat peas had been reached, after being gradually increased over a period of thirteen weeks.

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Some Antigenic Characteristics of *Salmonella Pullorum*

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THIS REPORT deals primarily with differences found in the antigenic balance of XII_2 and XII_3 antigens* in *Salmonella pullorum* cultures isolated from various sites in individual birds, as well as antigenic differences in cultures isolated from different birds from the same flock.

A total of 176 *S. pullorum* cultures isolated from 39 birds from ten different flocks is included in this report. Serum-agglutinin response of these birds is included.

The initial recognition by Younie[10] in 1941 of an antigenic variation in some strains of *S. pullorum*, as evinced by agglutinogenic response of infected birds, has been confirmed by Byrne,[1] Edwards and Bruner,[2,3] Edwards *et al.*,[4] Gwatkin,[5,6] Gwatkin and Bond,[7] Wright,[9] Younie,[11] and others. These workers have repeatedly demonstrated that serum of birds infected with the variant form of the organism may agglutinate variant form antigen and be negative to standard form antigen. Edwards and Bruner,[2,3] in 1946, reported agglutinin-absorption tests showing the somatic antigenic formula of *S. pullorum* to be IX XII, [XII_2] XII_3. (The brackets are used to indicate that the XII_2 antigen is variable.) Variant, or X, cultures were found to have a strong XII_2 antigen and a weak XII_3 antigen. Standard cultures had a weak XII_2 antigen and a strong XII_3 antigen. Thus, actually both XII_2 and XII_3 antigens are variable in approximately an inverse ratio. In various reports to date, symbols used to designate the antigenic composition of cultures, as well as of individual colonies, have not always been clear. Wright[9] has used plus and minus signs to designate the apparent relative balance of the XII_2 and XII_3 antigens. Slide-agglutination reaction was used as an indication of the relative amounts of the two antigens present. Thus, a variant form colony would be designated as XII_2 ++ XII_3 — (++ —); a standard form colony as XII_2 — XII_3 ++ (— ++). He recognizes the occurrence of colonies containing both of these antigens in varying proportions, some of which contain relatively large amounts of both antigens in apparent balance. These colonies are designated as XII_2 ++ XII_3 ++ (++ ++). Colonies containing both antigens in an apparent unbalanced condition were designated either as +— ++ or ++ +—. It is generally recognized that the sensitivity of various colonies and strains to agglutination by homologous absorbed serum is widely divergent. The comparative degree and rate of agglutination of a colony cell suspension by specific immune serum has been used by Wright[9] as an indication of the quantitative antigen content. Using this criterion of determination of antigen quantity, it would seem that a relatively insensitive standard form colony, for example, should be designated as XII_2 — XII_3 +— rather than using a double plus designation for all colonies containing detectable amounts of only one of these two antigens, regardless of sensitivity to agglutination.

Standard strains recently isolated by Edwards and Bruner[2,3] were found to contain both standard and variant form colonies. Variant strains, however, contained only XII_2 ++ colonies and were apparently stabilized in that form. Wright[9] reported finding 2 to 5 per cent of the individual colonies of standard strains 17, 19, and 20* to be other than XII_2 ++ in antigenic composition even after the long period of artificial cultivation of these strains.

Terms used to designate the antigenic form of cultures are also confusing. Edwards,** for convenience, defines a culture fairly well balanced in XII_2 and XII_3 antigens as being the normal form of the organism which is in active form variation. Wright and Edwards[9] have used the term "intermediate" for this same type of culture. It is recognized that agglutination by both XII_2 and XII_3 serums will be produced in cell suspensions of cultures composed of well-balanced numbers of standard and variant colonies, as well as from cultures in which the individual colonies contain considerable amounts of both antigens. The authors† have isolated at least one culture

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Since submitting this report for publication, the authors have accumulated additional data showing that at times birds infected with a nonvariant form of *S. pullorum* may, in a significant percentage of cases, yield serum which strongly agglutinates variant antigen and produces little or no agglutination in standard antigens.

*A double plus sign (++) following the antigen designation is used in this report to indicate that the particular cell suspension used was agglutinated only by that agglutinin but does not indicate rate or degree of agglutination.

*Isolated by Dr. L. F. Rettger, Yale University, in 1916, 1911, and 1917, respectively. These strains were accepted in 1931 by the Northeastern Conference of Laboratory Workers for producing antigen for routine pullorum testing by the tube-agglutination method.

**Personal communication.

†Unpublished data.

possessing both XII_1 and XII_2 antigens in approximately equal amounts as indicated by individual colony form-typing. This characteristic was found in all of 200 colonies form-typed during ten serial transfers over a two-month period. This evident stability suggests that the culture was not undergoing active form variation and, therefore, could not be accurately classified as either normal or intermediate.

Culture Procedure

Beef extract broth and agar were the main culture mediums used. Cultures made from sections of the intestinal tract and occasional contaminated cultures were incubated in tetrathionate broth for twenty-four hours before plating on MacConkey's agar. All grossly pathologic tissues were cultured in addition to routine culturing of the heart blood, gall bladder and a section of the liver, peritoneal fluid, oviduct, crushed ovary, and approximately ½-in. sections of the ileum and cecum. All cultures were incubated in broth for twenty-four hours before plating, except cultures of pathologic tissues which were made directly on agar plates. The spleen was cultured by incision and impression of the pulp directly on agar plates. Eggs cultured were held in an incubator for seventy-two hours before breaking into broth and incubating an additional forty-eight hours before plating.

Form-Typing Procedure

Absorbed immune serum was prepared as described by Wright and Edwards.[6] The first plate culture secured from an organ or tissue was used for form-typing. A cell suspension prepared from a large number of colonies from each culture was used for slide agglutination with absorbed serums. This procedure is referred to in this report as composite form-typing. Discrete colonies for individual colony form-typing were secured by plating a broth dilution of an inoculum prepared from a large number of colonies taken from the original plate culture. The number of culture passages was kept at a minimum (not more than two) to prevent, in so far as possible, the relative form-stabilization which most workers have noted to occur following artificial culture passage. All cultures isolated were form-typed by the composite method. At least 50 colonies of one culture isolated from each bird were typed by the individual colony method, as well as any culture which produced composite agglutination reactions differing from those of cultures isolated from other sites in the same bird. The accuracy of the results secured by colonial and composite form-typing was frequently checked during the course of the work by the authors independently form-typing a culture and comparing results. In all cases, the procedure appeared highly accurate within the

TABLE 1—Serum-Agglutination Reaction of Birds Selected from Flock E

Bird (No.)	Flock test 1 : 25 dil. 9/14/48 Std.	Var.	S. pullorum isolated	Tube-agglutination test on receipt of birds 9/28/48 Standard 10	20	40	80	Variant 10	20	40	80	1948 Date of necropsy	Tube-agglutination test at necropsy Standard 10	20	40	80	Variant 10	20	40	80
7664	0	2	—	0	0	0	0	2a	1a	1a	1f	10/11	0	0	0	0	1a	1a	0	0
7670	0	4	—	1f	0	0	0	2a	1a	1f	1f	10/21	1f	0	0	0	2a	2a	1a	1a
7673	0	4	—	0	0	0	0	3a	3a	2a	2a	10/20	0	0	0	0	3a	3a	3a	2a
7675	0	4	+	Serum unsatisfactory								10/11	4	3	3	3	4	4	2	1
7709	4	4	+	4	4	4	3	4	4	3	2	11/4	4	4	3	2	4	4	3	2
7716	4	0	+	4	4	2	1	4	1f	0	0	11/19	4	3	2	1	1a	0	0	0
7718	4	4	+	4	4	4	3	4	4	4	3	11/19	4	4	4	4	4	4	4	4
7762	0	3a	+	4	4	4	4	4	4	4	4	9/28	‡							
7794	1a	3a	+	Died from acute infection								9/28	‡							
7919	0	4f	+	Serum unsatisfactory								9/28	‡							
7925	4	2a	+	4	4	4	3	4	4	3	2	11/19	4	4	4	4	4	4	4	4
7936	0	3f	—	0	0	0	0	1a	1a	1f	1f	9/30	‡							
7952	0	3a	—	1a	0	0	0	0	0	0	0	9/30								
7973	4	1a	+	4	4	4	4	4	4	4	4	11/12	4	4	4	4	4	4	4	4
7976	0	4	+	4	4	4	4	4	4	4	4	10/7	4	4	4	4	4	4	4	4
7995*	4	2a	—	4	4	2	2	3a	1f	0	0	11/12	4	3	2	1	3	2	1	0
8088	0	4f	—	1f	0	0	0	2f	1f	1f	0	9/29	‡							
8146	0	3a	—	0	0	0	0	3	3	2	1	9/30	‡							
9020	0	3a	—	0	0	0	0	2f	1f	1f	0	9/28	‡							
9059	0	3f	—	0	0	0	0	1f	1f	1f	1f	9/30	‡							

*Typical lesions of *S. pullorum*, only direct cultures made.

‡Birds necropsied within two days of receipt, no agglutination tests made at that time.

0 = no agglutination; 1 = slight agglutination; 2 = partial agglutination; 3 = incomplete agglutination; 4 = complete agglutination; a = atypical; f = fine.

limits that the results are used for interpretation in this report. Very slight agglutination produced by the minor antigen of a colony or culture-cell suspension occasionally produced a slight disparity of results but did not appear significant in the evaluation of the data.

doubtful reaction to the variant antigen (table 1, birds 7716, 7925, 7973, and 7995). It appears significant that all of these birds showed typical lesions of *S. pullorum* infection on necropsy and that 3 of the 4 yielded *S. pullorum* cultures. No culture was se-

TABLE 2—Comparative Agglutination of Standard and Variant Antigens with Selected Serums

Bird (No.)	Standard antigen									Variant antigen				
	10	20	40	80	160	320	640	1280	2560	10	20	40	80	160
48368*	4	4	3	2	1	0				1a	0			
50630	4	4	4	4	4	3	2	1	0	4	3	2	1f	0
50197	4	4	4	3	1f	0				4	3	1f	0	0
90538*	4	2	1	0						1	1f	0		

*At necropsy twenty and thirty days later, respectively, serums of these birds were strongly positive to both antigens, and some cultures isolated showed a marked mixing of antigenic forms as shown in table 3.

RESULTS

In one adult flock included in this report (flock E), the disease was accompanied by a rather high mortality from acute infection. *Salmonella pullorum* was isolated from a dead bird sent to the diagnostic laboratory. Composite form-typing of this culture showed the presence of both XII_2 and XII_3 antigens. More than one half of the 100 individual colonies typed showed significant amounts of XII, antigen; most of these colonies were XII_2 + +. The remainder of the colonies were XII_3 + +. This flock was bled eighteen days later and each serum was tested with standard antigen (conference strains 17, 19, and 20) and with a variant antigen (Wright 296) in a 1 : 25 dilution. At this time, 81 of the 890 birds remaining in this flock reacted to the standard antigen with a sufficiently complete agglutination to be considered infected. Serums from 10 of these 81 birds did not agglutinate the variant antigen sufficiently to be considered positive, although most of them showed some slight atypical agglutination. Serums from 105 birds in this flock agglutinated variant antigen in a positive or highly suspicious manner. Serums from 164 additional birds in this flock showed a lesser degree of agglutination of variant antigen. Twenty birds (table 1) with varying types of serum-agglutination reactions were secured fourteen days after testing. In the interval between testing the flock and securing the selected birds, the owner reported a moderate mortality which appeared to be caused by acute *S. pullorum* infection. For this reason, some of the selected birds may have yielded cultures at necropsy which actually were not infected at the time of the first test.

Four of the 20 birds secured from flock E had shown an initial positive reaction to the standard antigen and a negative or

cured from bird 7995, but this was undoubtedly due to an error in culturing. Only direct plate cultures were made of the pathologic tissues; broth cultures were not made. Cultures isolated from each of the 3 birds showed a small percentage of colonies that were other than XII_3 + +, most of which were XII_2 + + (one to four colonies in 50 examined per bird). When received fourteen days after the initial flock test, serums from all 4 of these birds showed a positive titer to the standard antigen, two of these serums also showed a positive titer to the variant antigen, but serums from birds 7716 and 7995 produced significant agglutination only at 1:10 dilution of the variant antigen, and only slight agglutination at 1 : 20 dilution. At necropsy, sixty-six days after the initial flock test, bird 7716 was still positive to the standard antigen and essentially negative to the variant antigen. Bird 7995 developed a slightly higher titer to variant antigen, but it remained less than the titer to the standard antigen. Cultural results and agglutinin response of 11 of the 20 birds secured from flock E were not considered remarkable and are not specifically referred to in this report. The serums of 4 other infected birds from 3 flocks showed a markedly higher agglutination titer to the standard than to the variant antigen used (table 2). Antigenic characteristics of cultures isolated from these birds are shown in table 3.

In some infected birds, a wide variation was found in the antigenic balance of cultures isolated from different organs or sites as indicated by single colony form-typing (table 3). Bird 78554, for example, furnished an ovary culture agglutinated only by XII_3 serum; 49 of 50 individual colonies were XII_3 + +; the remaining colony was XII_2 + +. A culture isolated from the gall bladder and liver of this bird showed a

marked agglutination by XII_2 serum and only slight agglutination by XII_3 serum. Individual colony form-typing showed 25 of the 50 colonies examined to be XII_3 ++, 23 to be XII_2 ++, and the remaining two to be agglutinated by both XII_2 and XII_3 serums. A significant degree of this type of antigenic variability was found in cultures from 14 of the 39 infected birds. Eleven of these 14 birds were from four flocks which furnished other infected birds yielding cultures uniformly XII_3 ++, and containing only a few XII_2 ++ colonies. Three of the birds were from flocks from which only 1 positive bird was cultured. Cultures isolated from all sites of the remaining 25 birds were fairly well stabilized as XII_3 ++ and had an insufficient amount of XII_2 antigen to be detected by composite form-typing.

Some positive birds were held in individual cages, and all eggs laid were cultured. All positive cultures were form-typed. A marked variation in antigenic balance of cultures secured at different times from some birds was recorded. An example of this type of variation was bird 7718 which laid the first infective egg on Oct. 11, 1948. Composite form-typing showed the presence of both XII_2 and XII_3 antigens in approximately equal amounts. Twenty-two of 50 individual colonies were XII_3 ++, 12 were XII_2 ++, and the remaining 16 colonies contained both XII_2 and XII_3 antigens. Infective eggs, laid Oct. 22, 1948, and Nov. 6, 1948, showed a progressive decrease in the number of colonies containing XII_2 ++ antigen. A culture secured from the ovary at necropsy on Nov. 19, 1948, was agglutinated by XII_3 serum but not by XII_2. Only three of the 50 colonies examined were XII_2 ++; the remainder were XII_3 ++. A definite degree of this type of antigenic variability was noted in 4 of 8 birds from which infected eggs were secured over a period of three weeks or more.

Some cultures showing a sufficient mixing of XII_2 ++ and XII_3 ++ colonies to be readily detected by composite form-typing tended to stabilize as XII_2 ++ cultures, and predominantly XII_3 ++ colonies, after variable periods of growth and transfer on artificial mediums. In some cases, direct plate cultures secured from an organ were very strongly agglutinated by XII_2 serum and only slightly by XII_3 serum. After dilution and plating for individual colony form-typing, this serum agglutination would sometimes be reversed, and individual colonies would be predominantly XII_3 ++ (table 3, birds 48368 and 78554). This fact suggests the possibility that, in some birds, the infecting organisms may be predominantly XII_2 ++ and not be a true variant form because of instability and lack of purity of colonial form. If this is true, it is possible that birds infected with this form of *S. pullorum* may react weakly or not at all to standard form antigens and

TABLE 3—Antigenic Composition and Behavior of Selected Cultures

Bird (No.)	Site of culture isolation	Primary culture composite agglut.		Individual colony agglut. reaction (from broth dilution of primary culture)			After 30 days' growth on artificial mediums				
							Composite agglut.		Individual colony agglut.		
		XII_2	XII_3	Only XII_2	XII_2 and XII_3	Only XII_3	XII_2	XII_3	Only XII_2	XII_2 and XII_3	Only XII_3
48368	Follicular fluid	+	slight		5	195	•	•			
48368	Ileum and cecum	—	+		4	46					
48368	Peritoneum	slight	+	4	3	43					
48368	Oviduct	—	+		1	49	—	+	1		49
50608	Peritoneum	—	+	•	•	•					
50608	Follicular fluid I	—	+	•	•	•					
50608	Follicular fluid II	—	+	3		47	•	•			
50630	Peritoneum	—	+								
50630	Gall bladder and liver	—	+								
50630	Ova	—	+		1	49	—	+	1	1	48
78554	Ovary	—	+	1		49	—	+	9	4	87
78554	Peritoneum	—	+								
78554	Gall bladder and liver	+	slight	23	2	25	—	+	3		47
90197	Peritoneum	—	+								
90197	Ovary	—	+	1	1	48					
90538	Peritoneum	+	+	14	5	31					
90538	Ovary	—	+	1		49					
90538	Gall bladder and liver	+	slight	28	1	21					

*Insensitive.

be positive to variant antigens. Birds 7675, 7762, 7919, and 7976 (table 1) did show an initial positive or doubtful reaction to variant antigen and no reaction to the standard antigen; although, at a later date, 3 of these birds were positive to both antigens used, and cultures isolated from these birds were of a predominantly XII_3 ++ form. These types of reactions, however infrequently they may occur, emphasize the impossibility of accurately classifying the form of the infecting organism by the agglutinogenic response of the infected bird or by cursory examination of cultures isolated.

Many of the cultures showed a marked tendency to become insensitive to agglutination by absorbed serums after variable periods of growth on beef extract agar. In some cases, as few as two 48-hour transfers resulted in almost complete insensitivity to the slide-agglutination test (table 3, bird 50608).

SUMMARY

1) None of the cultures studied in this survey proved to be of the variant form of *Salmonella pullorum*.

2) In some *S. pullorum*-infected flocks, the ratio between the number of colonies containing XII_2 ++ antigen and those containing XII_3 ++ antigen, as indicated by primary culture isolations, varied widely between different infected birds in a flock as well as between different sites of isolation in individual birds. Cultures isolated from eggs of infected birds may show a variation in the balance of these antigens over a period of time.

3) In some *S. pullorum*-infected flocks in which the isolated colonial growth was predominantly XII_3++, the antigenic composition of cultures isolated showed considerable uniformity. No cultures containing only XII_3 ++ colonies were found.

4) In one flock studied, the serums from a significant number of birds produced positive agglutination of a standard strain antigen and little or no agglutination of a variant strain antigen. Three out of 4 of these birds, cultured later, proved to be infected. Similar agglutination reaction was produced by the serums of 4 infected birds from three other flocks.

5) It appears that serums of birds infected with a nonvariant form of *S. pullorum* may, at times, agglutinate variant antigens and not standard antigens.

6) Cultures which showed a marked mixing of XII_2 ++ and XII_3 ++ colonial forms at primary isolation showed a tendency to become predominantly XII_3 ++ forms following even short periods of growth and transfers on the artificial mediums used.

7) Sensitivity of XII_3 ++ forms to agglutination by homologous serum may be rapidly lost following growth on artificial mediums.

8) Birds infected with *S. pullorum* not of a variant form may yield cultures with almost any ratio between XII_2 and XII_3 antigens; this being a variable factor, concise classification of these forms is impracticable.

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Studies on Newcastle Disease

V. A Comparison of Newcastle Disease Antibody Titers in Blood and Egg Yolk as Measured by the Hemagglutination-Inhibition Test

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SINCE 1944, Newcastle disease has been reported from almost every part of the United States[1] and has become a serious threat to the poultry industry. One of the prime factors in effective disease control is a practical, accurate method of diagnosis. Schmittle and Millen[3] described a method for using egg yolk in place of serum in the hemagglutination-inhibition (HI) test. The present report describes an improved technique for the yolk-HI test, and compares serum-HI antibody titers with egg-yolk titers in the same hens.

MATERIALS AND METHODS

Tortor Furens (Newcastle Disease Virus).—The strain of *Tortor furens* used in the HI test was California 11914 obtained from Dr. C. A. Brandly of the University of Wisconsin, Madison, in July, 1946, and subsequently propagated in chicken embryos at the University of Illinois, Urbana. The virus was contained in blood-free allantoic fluid from chicken embryos which had died of Newcastle disease. Before each series of tests, the hemagglutination titer was determined for the allantoic fluid in order to dilute the fluid so that each 0.25 ml. contained ten hemagglutinating units.

Chicken Red Blood Cells.—Several mature chickens whose red blood cells agglutinated easily were maintained as blood donors. Blood samples were collected from the wing vein, using a 5-ml. syringe containing 0.5 ml. of 2 per cent sodium citrate. The red cells were washed and suspended in sufficient 0.85 per cent sodium chloride to make a 1 per cent suspension. The red blood cells were discarded after four days.

TABLE 1—Protocol of Ingredients Required for *Beta* Hemagglutination-Inhibition Test

	Tube 1	Tubes 2 thru 9	Tube 10	Tube 11	Tube 12
Serum or yolk	0.25 ml. of 1:5 dilution	0.25 ml. of serial twofold dilution from 1:10 to 1:1280	0.25 ml. of 1:5 dilution	None	None
Tortor furens	0.25 ml. 10 hemagglutinating units	0.25 ml.	None	0.25 ml.	None
0.85 per cent saline	None	None	0.25 ml.	0.25 ml.	0.50 ml.
1 per cent suspension of r. b. c.	0.25 ml.	0.25 ml.	0.25 ml.	0.25 ml.	0.25 ml.

Treatment of Yolk.—To obtain yolk, eggs were broken and the yolk was withdrawn with a 14-gauge hypodermic needle into a 2-ml. Luer lock syringe. One and one-half milliliters of yolk were placed in a 13 by 130-mm. test tube containing 6 ml. of 0.85 per cent sodium chloride solution, and the mixture was shaken. Two milliliters of ethylene dichloride and 1 ml. of diethyl ether were added, and the contents of the tube were thoroughly mixed by shaking and inverting. The mixture was

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incubated at 37 C. overnight and then centrifuged. The ethylene dichloride fraction collected at the bottom of the tube and was separated from the aqueous fraction by a fatty ring. The aqueous fraction was substituted for serum in the HI test.

The above technique was described by Schmittle and Millen.[7] Because its results were not always consistent, an attempt was made to improve the technique by varying the temperatures and incubation times, after adding ethylene dichloride and ether to the diluted egg yolks. The most satisfactory procedure was found to be incubation in a 50 C. water bath for four hours followed by refrigeration at 4 C. for eight hours to overnight.

Beta Hemagglutination-Inhibition Test.—The method used for the *beta* HI test was that reported by Brandly *et al.*[1] The serums and aqueous yolk extracts were diluted with saline in serial twofold dilutions from 1 : 5 through 1 : 1,280 in chemically clean 13 by 130-mm. test tubes. Next, 0.25 ml. of each dilution of serum and yolk was transferred to 10 by 75-mm. serologic tubes. To each tube were added 0.25 ml. of virus suspension containing ten hemagglutinating units and 0.25 ml. of a 1 per cent suspension of chicken red blood cells. The test was set up as shown in table 1. Tube 10 was a serum control, tube 11 a virus control, and tube 12 a saline control. The virus was added to the serum or yolk first; then the red blood cell suspension was added. The tubes, having been placed in a rack, were shaken thoroughly and allowed to incubate at room temperature (22 to 27 C.). The rack was placed on a stand which had a mirror set at a 35-degree angle beneath the tubes. A light source was placed over the tubes so that the image of the patterns on the bottoms of the tubes could be seen in the mirror. Following incubation for fifteen minutes, the tubes were observed every five minutes until the red blood cells in the serum control tubes had settled out on the bottom in compact disks. The highest dilution of serum or yolk in which there was complete inhibition was considered the HI titer. Complete inhibition by serum or yolk diluted 1 : 5 or more was considered as positive for HI antibodies.

Experimental Animals.—Three groups of hens were used as experimental animals.

1) Hens Not Previously Exposed to *Tortor Furens.*—Forty-nine White Rock pullets were

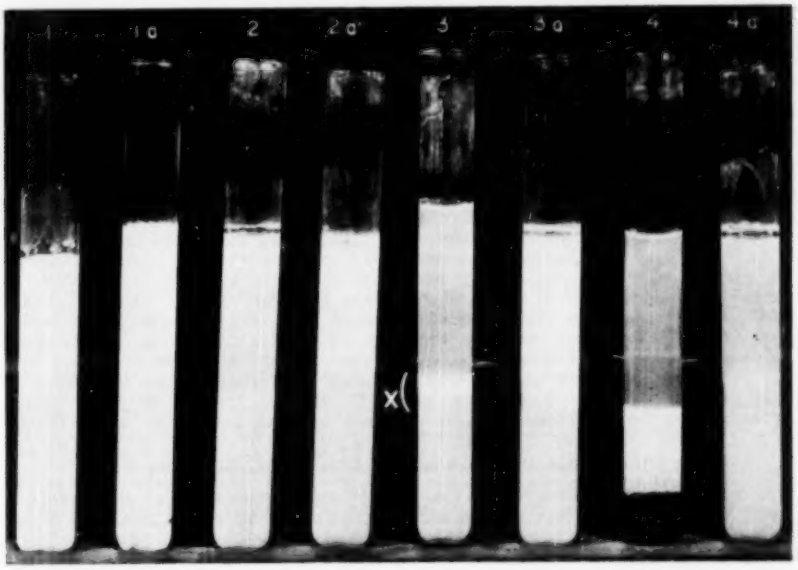

Fig. 1—Effect of contaminating egg albumen on appearance of yolk extracts in processing egg yolk for HI test. Tube 1—diluted egg-yolk mixture following addition of ethylene dichloride and ether. Tube 2—egg-yolk mixture after incubation at 50 C. for four hours. Tube 3—egg-yolk mixture after refrigeration at 4 C. for eight hours (x denotes cold precipitate). Tube 4—egg-yolk mixture after centrifugation. The slightly cloudy upper layer is the aqueous fraction used in the HI test. Tubes 1a, 2a, 3a, and 4a are similar to tubes 1, 2, 3, and 4 except they have been contaminated with egg albumen. Note cloudiness of the aqueous layer.

purchased from a flock determined to be free of Newcastle disease by history and serum-HI tests.

2) Hens Which Had Gone Through an Outbreak of Newcastle Disease.—Sixty-five Leghorn hens were purchased from a flock in which a natural outbreak had been diagnosed by the University of Illinois Diagnostic Laboratory three months before.

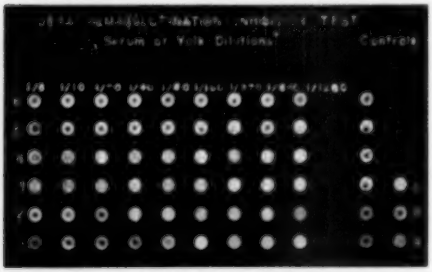

Fig. 2—Comparison of serum- and yolk-HI titers from the same hens. Rows 1, 3, and 5 are serum dilutions from 3 different hens, rows 1 and 5 being positive while row 3 is negative. Rows 2, 4, and 6 are corresponding yolk dilutions; row 2 and 6 are positive and row 4 negative. Compact circular masses of red blood cells denote absence of agglutination. Ring deposits of red cells and clear tubes represent hemagglutination. The rings of cells in the tubes on the left side of row 4 illustrate the result of incubating several minutes beyond the optimum reading time. Tube 10, the tube to the right of the tube containing the 1 : 1,280 dilution is the positive serum or yolk control. Tubes A and C are virus controls; tube A contains virus from the first portion of the virus suspension, while tube C is virus from the last portion of the suspension. Tube B is the saline control.

3) Hens Previously Exposed Artificially to *Tortor Furens.*—Sixty-eight hens were secured from a vaccination experiment. They had been either vaccinated with a living, duck embryo-propagated vaccine or challenged with living *T. furens.* The viruses used for vaccination were either the California strain 11914 or a strain isolated from the University of Illinois poultry flock. Since there was no correlation between the type of vaccine used in the experiment and differences in results, further details about the vaccines are not included.

As soon as the hens from all three groups came into production, eggs and blood samples were collected on the same day from each hen. Hemagglutination-inhibition antibody titers were determined for each serum sample and compared with the HI titers of the corresponding yolks.

RESULTS

Refrigeration.—Refrigeration at 4 C. for eight hours rendered the aqueous layer more suitable for testing because it layered out a "cold precipitate" on top of the fatty ring (fig. 1). If the cold precipitate were allowed to remain in solution, it caused slight, nonspecific virus inhibition. Eight hours' refrigeration at 4 C. was found to be the minimum time required to bring down the cold precipitate. For the tests described herein, the tubes were usually allowed to remain at 4 C. overnight.

Effect of Albumen.—In removing yolk from the yolk sac, it was found essential that no egg albumen be included. If the yolk were contaminated with albumen, its aqueous fraction was very cloudy after incubation (fig. 1). The albumen inhibits virus agglutination in known HI-negative eggs (table 2). Lanni and Beard[2] report inhibition by egg white of hemagglutination by swine influenza virus.

Comparison of Newcastle Disease Antibody Titers in Blood and Egg Yolk as Measured by the Beta Hemagglutination-Inhibition Test.— 1) Serums and yolks from 49 hens with no history of Newcastle disease were negative to the *beta* HI test.

2) Serums and yolks from 65 hens that had gone through a natural outbreak of Newcastle disease were all positive to the *beta* HI test. In 36 (55.4%) hens, the serum titer was the same as the yolk titer. In 28 (43.1%) hens, the serum titer was twice the yolk titer. In 1 (1.5%) hen, the serum titer was 32 times the yolk titer.

3) Results for the 68 hens which had been exposed artificially to *T. furens* are

TABLE 2—The Effect of Albumen on the Yolk-Hemagglutination-Inhibition Test

No. of eggs tested		Appearance of aqueous layer		Results of HI tests	
		Cloudy	Clear	Satis.	Unsatis.*
56	Before addition of albumen	0	56	56	0
	After addition of albumen	56	0	14	42

*Unsatisfactory HI tests were those in which a nonspecific inhibition occurred with known HI-negative eggs.

shown in table 3. The titers varied in different hens from 1 : 5 through 1 : 640.

DISCUSSION

A comparison of the HI antibody titers of the yolks with those of the serums from the same hens in the present study indicates that there is a close correlation between the two. On the basis of these results, it seems reasonable to assume that yolk can be substituted for the serum in diagnosing previous exposure to *T. furens*.

The time at which the test was read was found to be important, particularly with the aqueous yolk extract. After a fifteen-minute incubation period, the tubes should be observed at five-minute intervals until unagglutinated masses or "buttons" of red blood cells form in the control tubes. In yolks which have no inhibiting antibodies, the agglutinated cells settle more rapidly and adhere, temporarily at least, to whatever portion of the hemispherical bottom of the tube they first contact. As the cells accumulate, a characteristic ragged edge appears around the thin deposit of cells. This ragged edge, which is caused by slipping of agglutinated cells to the bottom of the tubes, takes place more rapidly at higher temperatures. If the tubes are incubated too long at room temperature before being read, it may be found that what was originally a typical negative tube may contain a deposit of red blood cells which at a casual glance may have the appearance of a positive reaction (fig. 2). If the tests are not read at the optimum time, one can distinguish between the deposit of agglutinated cells and the compact mass of unagglutinated cells by tilting the rack of tubes horizontally. The masses of unagglutinated cells run smoothly down onto the side of the tube, whereas the deposit of agglutinated cells does not stream except after the tubes have been allowed to incubate for more than an hour at room temperature.

If properly conducted, the yolk-HI test is apparently a reliable test and may have several applications and possible advantages.

1) It can be used to detect previous exposure of hens to *T. furens* following either a natural outbreak of the disease or vaccination with a living vaccine.

2) Eggs are easier to collect from hens than blood samples and are more resistant to freezing and heat.

3) Bacterial putrefaction, commonly seen in blood samples submitted to the laboratory, is largely avoided in fresh eggs.

TABLE 3—Serum- and Egg-Yolk- *Beta* Hemagglutination-Inhibition Titers of Hens Recovered from Experimental Newcastle Disease

Relation of serum and yolk titers	No. of hens	Per cent of hens
A—Both serum and yolk positive	63	92.6
1—Serum titer one-half the yolk titer	(6)	(8.8)
2—Serum and yolk titers equal	(38)	(55.8)
3—Serum titer twice the yolk titer	(15)	(22.0)
4—Serum titer 4 times the yolk titer	(2)	(3.0)
5—Serum titer 8 times the yolk titer	(1)	(1.5)
6—Serum titer 16 times the yolk titer	(1)	(1.5)
B—Serum positive and yolk negative*	3	4.4
C—Both serum and yolk negative	2	3.0
Total	68	100.0

*Serum titer 1 : 20 or less.

SUMMARY

1) An improved technique is described for extracting lipids from egg yolk so that the hemagglutination-inhibition (HI) test for Newcastle disease can be carried out on eggs. The yolk is mixed with ethylene dichloride and ether, and the mixture allowed to stand for four hours at 50 C. and then for eight hours to overnight at 4 C.

2) A comparison was made of egg-yolk- and serum-*beta* HI titers of 49 hens with no history of Newcastle disease and of 136 hens which had been either naturally or experimentally infected with *Tortor furens* (Newcastle disease virus). Both the serums and yolks of the hens which had not had the disease were negative to the HI test. The serums and egg yolks of 96.3 per cent of the exposed hens were both positive; those of 1.4 per cent were both negative. In 2.3 per cent, the serum was positive and the yolk negative. In 54.4 per cent of the hens, the serum and egg-yolk titers were the same; in 37.5 per cent, the serum titer was higher than the yolk titer; in 4.4 per cent, the yolk titer was higher.

3) Contamination of the yolk with albu-

men caused nonspecific hemagglutination inhibition.

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Electron Micrographs of the Mouse-Adapted Newcastle Disease Virus

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A STRAIN OF mouse-adapted Newcastle disease virus[1] (NDV), which had been carried through 17 subcultures in this species, was used in this experiment.

Ten mice were injected intracerebrally with the seventeenth subculture and were sacrificed upon showing symptoms of Newcastle disease. The brains were removed aseptically and ground to a 10 per cent suspension in physiologic saline solution. The mouse-brain suspension was then subjected to centrifugation at 1,000 r.p.m. for five minutes. The supernatant liquid was removed and centrifuged again at 50,000 r.p.m. for two hours in a Spinco ultracentrifuge with a change in temperature of 1 degree. The supernate was discarded, and the sediment was resuspended in 0.5 cc. of physiologic saline solution. (The presence of virus was demonstrated by the intracerebral injection of 6 four-week-old Swiss albino mice, of which 4 developed typical NDV-infection symptoms and died.) A small drop of this virus suspension was then placed on parlodion film supports. After all the excess fluid had been removed with a fine capillary pipette, the films were dried. Some were shadowed with chromium[2,3,4] and examined in an RCA type EMU electron microscope, as shown in figure 1; and some were examined unshadowed, as shown in figure 2. The micrographs showed that the spermlike forms predominate, such as reported by Bang,[5,6] and Cunha and his coworkers,[7] as they found in Newcastle disease-infected egg-allantoic fluid. Reagan *et al.*[8] also found spermlike forms in the hamster-adapted NDV (California strain 11,914)

Fig. 1—Mouse-adapted Newcastle disease virus with 10 mg. of chromium at a tangent of ¾. x 72,900.

Fig. 2—Mouse-adapted Newcastle disease virus unshadowed. x 57,600.

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when the virus particles were suspended in normal saline. Also, spermlike forms were found after injecting hamster-infected Newcastle disease brain- and mouse-infected

Fig. 3—Mouse-adapted Newcastle disease virus shadowed with 10 mg. of chromium at tangent ¾. x 69,600.

Newcastle disease brains, after recultivating back into 10-day chicken embryos.[9]

The mouse-adapted NDV tails seemed to show small segments in the electron photographs at this high magnification.

SUMMARY

Electron micrographs of saline suspended, mouse-adapted Newcastle disease virus (NDV), after being subjected to concentration in the Spinco ultracentrifuge, showed very little difference from particles observed in saline suspension of the egg-adapted NDV California strain 11,914, except for the small segments observed in the tail. The segments of the tail seemed to show up much better without the chromium shadowing.

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Toxicity of Chlordan to Growing Chickens

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INTEREST IN the resistance of chicks to chlordan poisoning was increased as a result of a recent floor-brooding experiment conducted at the University of Hawaii Agricultural Experiment Station. It was observed that chicks died in large numbers when brooded on litter (bagasse) treated with a spray containing chlordan, DDT, and lethane. When the toxicities of these insecticides to chicks were compared, it was found that chicks fed chlordan died earlier and in less time than those fed DDT. In a paper recently published,[1] it was reported that chlordan, at 0.50 and 0.25 per cent of total ration, was toxic to 7-day-old chicks. This investigation was undertaken to determine the minimum lethal dose of chlordan for young chicks and to study its relative toxicity to growing chickens.

PROCEDURE

New Hampshire chicks from the same matings were reared to 7, 21, 63, and 112 days of age, at which time they were fed chlordan. The chicks were fed standard chick starter and grower mashes, and the chlordan was mixed in powder form into these rations. The number of chicks and the percentages of chlordan fed in this study are shown in table 1.

The 7-day-old chicks were fed chlordan for fourteen days, while the other three age groups received this insecticide for twenty-one days.

All chicks that died were submitted to Dr. H. E. Adler for postmortem examination and bacteriologic culture.

Dr. Adler reported that all bacteriologic cultures of the chicks that died were negative.

RESULTS

Chlordan killed all 7-day-old chicks when fed at levels ranging from 0.10 to 0.25 per cent. There was practically no difference in time to earliest death or distribution of death between the lots fed these concentrations. However, as may be seen in table 2, 0.05 per cent chlordan failed to kill all chicks. During the fourteen-day feeding trial, two thirds of this group died from chlordan poisoning. These data indicate that chlordan is very toxic to 1-week-old chicks when fed in concentrations as low as 0.05 per cent of total ration.

Tolerance to chlordan poisoning was greatly increased in chicks 21 and 63 days old. At the 0.10 per cent concentration, 95.2 per cent of the 7-day-old chicks died within five days, whereas, only 10 per cent of the 21-day-old chicks were dead at that time. Despite the increased resistance of the 21-day-old chicks to poisoning, chlordan was toxic to all chicks at either 0.10 or 0.25 per cent concentration within fourteen days (table 3). The 63-day-old chicks were markedly more resistant than the 21-day-old chicks. On 0.25 per cent chlordan, 100 per cent of the 21-day-old chicks died within eight days but only 20 per cent of the older chicks were dead at that time. Chlordan was lethal to all 63-day-old chicks within nineteen days when fed at either 0.50 or 0.25 per cent of the total ration.

The 112-day-old chickens were slightly more resistant to chlordan poisoning than the 63-day-old birds. The earliest death in the latter group on 0.25 per cent chlordan occurred at four+ days as compared with

TABLE 1—Experimental Design of This Study

Age of chicks (days)	Total no. of chicks	$\bar{X}$ body weight	Percentage chlordan in rations*						
7	126	64.6 Gm.	0.0	0.05	0.10	0.15	0.20	0.25	
21	80	181.6 Gm.	0.0	0.05	0.10			0.25	
63	30	1006.0 Gm.	0.0					0.25	0.50
112	24	2238.2 Gm.	0.0					0.25	0.50

*Dowklor, 40 per cent wettable: containing 24 per cent octochloro-4, 7 methano-tetrahydroindan, and 16 per cent related compounds.

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The writers are indebted to Dr. H. E. Adler, Pathology Laboratory of the Territorial Board of Agriculture and Forestry, Honolulu, T.H., for postmortem examinations of all birds that died during this investigation.

At present, Dr. Adler is at the College of Veterinary Medicine, State College of Washington, Pullman.

eight+ days for the 112-day-old birds. All birds succumbed to both levels of chlordan within eighteen days (table 3).

TABLE 2—Mortality Distribution Among Lots of 21, 7-Day-Old Chicks Fed Various Concentrations of Chlordan

No. days on chlordan	Percentage chlordan in rations					
	0.0	0.05	0.10	0.15	0.20	0.25
1	0	0	0	0	0	0
2	0	0	2	4	4	4
3	0	2	10	12	12	14
4	0	1	3	2	2	2
5	0	2	5	3	2	0
6	0	0	1	0	1	0
7	0	1	0	0	0	1
8	0	0	0	0	0	0
9	0	1	0	0	0	0
10	0	2	0	0	0	0
11	0	5	0	0	0	0
12	0	0	0	0	0	0
13	0	0	0	0	0	0
14	0	0	0	0	0	0
Total mortality	0	14	21	21	21	21
	21	21	21	21	21	21

The primary lesions of chicks that died, in all age groups and on the different levels of chlordan, were in the heart. There was an excessive quantity of fluid in the pericardial sac, enlargement and distortion of the heart, and engorgement of the coronary vessels. Occasionally, there were petechial hemorrhages of the myocardium. These lesions were noted after three days of chlordan feeding in the 21-day-old group, after five days in the 63-day-old group, and after nine days in the 112-day-old group. No chicks were sacrificed to determine the time of onset of these lesions.

DISCUSSION

The tolerance of chicks to chlordan poisoning increased progressively in the different age groups. Using the 0.25 per cent chlordan ration as a basis of comparison, the earliest deaths occurred at one+ days in the 7-day-old group, two+ days in the 21-day-old group, four+ days in the 63-day-old group, and eight+ days in the 112-day-old group. It should be remembered that these results were obtained under continuous feeding of rations containing chlordan. The tolerance of growing chicks may be greater when they are exposed to a single oral administration of chlordan. In a supplementary study, 9-day-old chicks were given 6.8 mg. of chlordan suspension in water, and 112-day-old cockerels were treated with 4.0, 2.0, or 1.0 Gm. of this insecticide in water suspension. The highest quantity for each age group was calculated on the basis of the feed consumption of the controls to the time of earliest mortality of the experimental chicks. The cockerel that received 4.0 Gm. of chlordan died on the fifth day; the cockerel that received 2.0 Gm.

TABLE 3—Mortality Induced by Various Levels of Chlordan Fed to 21-, 63-, and 112-Day-Old Chicks

No. days on chlordan	Age of experimental animals when chlordan was fed									
	21 days				63 days			112 days		
	Percentage chlordan in ration				Percentage chlordan in ration			Percentage chlordan in ration		
	0.0	0.05	0.10	0.25	0.0	0.25	0.50	0.0	0.25	0.50
1	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0
3	0	0	0	2	0	0	0	0	0	0
4	0	0	1	5	0	0	0	0	0	0
5	0	0	1	6	0	1	0	0	0	0
6	0	1	8	4	0	0	0	0	0	0
7	0	0	3	2	0	0	0	0	0	1
8	0	2	2	1	0	1	1	0	0	1
9	0	1	1	0	0	1	3	0	1	0
10	0	0	2	0	0	2	1	0	1	0
11	0	1	0	0	0	0	1	0	1	1
12	0	1	0	0	0	2	0	0	0	0
13	0	2	0	0	0	1	2	0	1	1
14	0	0	1	0	0	0	0	0	1	0
15	0	0	0	0	0	0	0	0	1	2
16	0	0	0	0	0	0	1	0	0	1
17	0	1	0	0	0	1	0	0	2	0
18	0	3	0	0	0	0	0	0	0	1
19	0	1	0	0	0	0	1	0	0	0
20	0	0	0	0	0	0	0	0	0	0
21	0	1	1	0	0	0	0	0	0	0
Total mortality	0	14	20	20	0	8	10	0	8	8
	20	20	20	20	10	10	10	8	8	8

showed very little effect, and the other two groups did not show any symptom of affliction at the end of five days. It would appear, therefore, that the use of sprays containing chlordan becomes less hazardous as chicks increase in body size, and as care is taken to prevent a residue that may be consumed over a period of days. It should be realized, nevertheless, that even 112-day-old chicks were killed in this experiment; therefore, sprays containing chlordan should be used prudently.

Chlordan should be used with extreme caution in areas accessible to young chicks. The spraying of litter or walls near the floor creates a hazard to young chicks because they are very susceptible to poisoning from the consumption of chlordan. The use of the lowest recommended concentration would increase safety in the use of this insecticide near young chicks and, at the same time, reduce the cost of spraying on poultry farms.

SUMMARY

Chlordan, when fed at concentrations ranging from 0.10 to 0.25 per cent of total ration, proved poisonous to 1-week-old chicks. At these levels, the earliest deaths were recorded within one+ days following the addition of chlordan to the ration. Chlordan, when fed at a concentration of 0.05 per cent of the total ration, killed 66.6 per cent of 1-week-old chicks within fourteen days.

Resistance to chlordan poisoning increased markedly in the several age groups. Using the 0.25 per cent chlordan concentration as a basis of comparison, it was observed that the earliest deaths occurred as follows: one+ days for the 7-day, two+ days for the 21-day, four+ days for the 63-day, and eight+ days for the 112-day-old chicks. This level of chlordan was toxic to all age groups when fed for twenty-one days.

The primary lesions noted in this study were in the heart. There was an excessive quantity of fluid in the pericardial sac, enlargement and distortion of the heart, and engorgement of the coronary vessels.

It is recommended that the use of chlordan be restricted to areas not accessible to young chicks.

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Toxicity of Chlordan to Laying Pullets

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LAYING CHICKENS are maintained almost exclusively on wire floors in the Hawaiian Islands; this system of management being reasonably effective in preventing them from feeding on the arthropod carriers of poultry parasites. However, on poultry farms where poultry manure collects under the cages or laying pens, and the farms are insanitary otherwise, heavy concentrations of burrowing roaches, cockroaches, beetles, and flies are found. On such farms, chickens frequently become infected with the eyeworm, *Oxyspirura mansoni*, and various tapeworms. Kartman *et al.*[1] have reported that chlordan is effective against roaches and beetles; and this insecticide in combination with DDT is now being routinely used in the control of insects at the poultry farm of the Hawaii Agricultural Experiment Station. Recently, Rosenberg *et al.*[2] reported that young chicks were highly susceptible to chlordan poisoning. In a subsequent paper,[3] it was reported that the tolerance to this insecticide greatly increased in growing chickens from 3 to 16 weeks of age. This investigation on the toxicity of chlordan to laying pullets was undertaken to complete the series of studies, since no papers dealing with the subject were found.

A comparable study on the toxicity of DDT to laying birds was reported by Rubin *et al.*[4] They observed that as little as 0.062 per cent of DDT (in peanut oil) in the diet for twelve weeks reduced egg production and hatchability.

EXPERIMENTAL

Twenty mature pullets in their fifth month of egg production were assigned to five lots of 4 birds each, according to rate of lay and body size. As shown in table 2, the heaviest lot received the highest concentration of chlordan, and the lower concentrations were assigned in descending order of average body weights. Chlordan was substituted on a dry-weight basis in a standard egg-laying ration so that the experimental diets contained 0.00, 0.05, 0.15, 0.25, and 0.50 per cent of this insecticide. The laying birds were maintained in individual laying cages and were fed these rations for twenty-eight days. Data were recorded every seven days on feed consumption and body weight, and daily observations were made on the response of the birds to these treatments. All birds in production were inseminated weekly with pooled semen. The chickens that died and all survivors were autopsied.

RESULTS

Chlordan depressed the chickens' ability to lay when fed at 0.50, 0.25, and 0.15 per cent of the total ration. The birds fed 0.50 per cent chlordan completely stopped laying within five days; those fed 0.25 per cent ceased production between eight and fourteen days; and the chickens receiving 0.15 per cent steadily dropped in rate of lay during the 28-day trial. As shown in table 1, the average egg production of the lot receiving 0.05 per cent chlordan was 19.7 per cent lower during the fourth week of this study than that during the twenty-eight days preceding the chlordan feeding. Since the production of the controls dropped 20.2 per cent during this period, it may be concluded that chlordan, when fed at the rate of 0.05 per cent, did not adversely affect egg production.

There was a positive relationship between the quantity of chlordan fed and loss in body weight. Following twenty-eight days on chlordan diets, the average body weight losses for the lots fed 0.05, 0.15, 0.25, and 0.50 per cent chlordan were, respectively, 0.50, 1.23, 1.17, and 1.85 lb. The control birds lost 0.05 lb. during this time. The weekly changes in average body weights of the five lots may be seen in table 2.

Chlordan also depressed feed consumption in accordance with the quantity fed. As may be seen in table 3, the birds fed 0.50 and 0.25 per cent chlordan reduced their feed consumption during the first week, and feed consumption in all lots receiving chlordan progressively declined to the end of the experiment. The birds receiving the least chlordan showed the smallest decline in feed consumption, and they were able to maintain body weight and lay eggs almost as well as the controls.

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Because fertility was low, it was not possible to procure an adequate record of the effect of chlordan on the developing embryo. The pullets receiving 0.50 and 0.25 per cent chlordan quickly ceased production, thereby tending to further restrict this phase of the molt was observed at the end of the first week, and molting progressively increased to the end of the trial; all but 1 chicken were out of production at the end of twenty-eight days; (d) 0.05 per cent lot—slight molting was observed on the thirteenth day;

TABLE 1—The Effect of Chlordan* on Egg Production

Percentage chlordan in ration	Average egg production of laying birds				
	$\bar{X}$ production during 28 days preceding test (%)	0-7 days (%)	8-14 days (%)	15-21 days (%)	22-28 days (%)
Control	77.3	76.1	57.1	52.3	57.1
0.05	62.5	67.8	64.3	50.0	42.8
0.15	61.6	42.8	25.0	25.0	7.1
0.25	66.9	50.0	17.8‡	0.0	0.0
0.50	68.8	46.4†	0.0	0.0	0.0

*Dowklor, 40% wettable: containing 34% octochloro-4, 7 methano-tetrahydroindan, and 16% related compounds. †Birds out of production at the end of 5 days. ‡Birds out of production at the end of 14 days.

study. Seventy-five per cent of the fertile control eggs hatched, whereas 76.4 per cent hatched from the 0.05 per cent chlordan-fed group. From these limited data, it was concluded that the eggs laid by the pullets receiving 0.05 per cent chlordan hatched as well as those laid by the controls. The number of fertile eggs laid by the other lots were too few to warrant any conclusion.

Antemortem inspection revealed the following symptoms of affliction: (a) 0.50 per cent lot—completely out of production on the fifth day and molting was observed on the sixth day; at the end of the first week, their combs began to shrivel; a heavy molt followed and the birds became progressively poorer in condition; by the end of this experiment, there was marked loss of body weight; (b) 0.25 per cent lot—a slight molt was observed on the sixth day and however, egg production for this group was almost as good as that of the controls throughout the test; aside from molting, this group showed no other disturbance. Since any condition that affects the general circulation is manifested by molt and cyanosis of the comb, it may be concluded that the syndrome described above was nonspecific.

Only 2 chickens died during this study. A pullet fed 0.25 per cent chlordan died on the twenty-seventh day and, on autopsy, a distorted heart and excess pericardial fluid were observed. The other chicken, a member of the 0.50 per cent lot, died on the twenty-first day and showed distortion of the heart, engorgement of the coronary vessels, and a distended pericardium containing a large amount of straw-colored fluid.

TABLE 2—The Effect of Chlordan on Body Weight

Percentage chlordan in ration	Average body weights of laying birds					
	Start of experiment (lb.)	7 days (lb.)	14 days (lb.)	21 days (lb.)	28 days (lb.)	Total loss (lb.)
Control	4.67	4.43	4.70	4.80	4.62	0.05
0.05	4.90	4.73	4.63	4.68	4.40	0.50
0.15	4.93	4.49	4.25	4.05	3.70	1.23
0.25	4.97	4.65	4.40	3.95	3.80	1.17
0.50	5.03	4.34	3.85	3.68	3.18	1.85

definite signs of molt were recorded on the tenth day; these birds ceased egg production within fourteen days and henceforth showed the same syndrome described for the 0.50 per cent lot; (c) 0.15 per cent lot—the birds in this group were highly variable in their response to chlordan; a slight

All survivors were autopsied at the end of the 28-day feeding period. The lesions of chlordan intoxication varied directly with the concentration used. At the lowest dosage (0.05%), no symptoms were seen on macroscopic examination. Birds fed 0.15 per cent chlordan showed blanching of the combs

and wattles and, aside from shrunken ova in several, their organs appeared normal. At the two highest levels (0.25 and 0.50%), there was marked shriveling and cyanosis of the combs and wattles, shrunken ova, nonfunctional oviducts, and emaciation. The 0.50 per cent chlordan-fed group exhibited the greater loss of body flesh.

DISCUSSION

The laying pullets in this experiment were highly refractory to chlordan poisoning. None of the birds receiving 0.05 or 0.15 per cent chlordan died despite continuous consumption during a 28-day period. The lots receiving 0.25 and 0.50 per cent survived without any mortality for twenty-seven and twenty-one days, respectively. The group receiving 0.50 per cent chlordan completely ceased egg production within five days and began to molt. The feed consumption steadily dropped and body weight losses were marked. At no time, however, did these birds exhibit symptoms that might be specifically attributed to chlordan intoxication even though one could observe their inactivity, shriveling and cyanotic combs, and heavy molt. The group receiving 0.25 per cent chlordan exhibited the same syndrome, although the onset was somewhat retarded. Similarly, the group receiving 0.15 per cent chlordan showed a comparable syndrome, although 1 bird was laying at the end of the feeding trial. The birds in this group were in fairly good condition at autopsy.

The group receiving 0.05 per cent chlordan were not affected to any great extent. These birds continued to lay nearly as well as the controls and at no time showed any symptoms of affliction; nor were any gross lesions observed on postmortem examination. It is unlikely that anyone could have known from the appearance of this lot that they had been ingesting chlordan.

The only gross pathognomonic lesions observed in the 2 pullets that died were in the heart. This finding is in agreement with that reported by Rosenberg *et al.*[2] on the effect of chlordan on growing chickens.

From the data procured in this study, it may be concluded that chlordan can be used in recommended concentrations around laying chickens without adverse effects, provided caution is exercised against leaving a residue that may be ingested over a prolonged period. The 0.05 per cent lot consumed 1.35 Gm. of chlordan during the duration of this experiment without adverse effects being noted.

These findings raise a question regarding the amount of chlordan that may be stored in chickens' tissues and eggs. Can chickens, inadvertently permitted to consume this insecticide, store sufficient chlordan to imperil the well being of persons who may consume their eggs and carcasses?

SUMMARY

1) Laying pullets responded to chlordan feeding by molting, ceasing egg production, losing body weight, and reducing feed consumption. This response was increased in accordance with the quantity fed. The lot receiving 0.50 per cent chlordan completely went out of production within five days; the lot receiving 0.25 per cent ceased laying within fourteen days; those receiving 0.15 per cent lowered production 54.5 per cent within twenty-eight days; whereas, the lot receiving 0.05 per cent continued to lay nearly as well as the controls.

TABLE 3—Feed and Chlordan Consumption During the 28-Day Trial

Percentage chlordan in ration	Ratio of chlordan to body weight at start of experiment	0-7 days		8-14 days		15-21 days		22-28 days	
		Feed consumed (Gm.)	Chlordan consumed (Gm.)	Feed consumed (Gm.)	Chlordan consumed (Gm.)	Feed consumed (Gm.)	Chlordan consumed (Gm.)	Feed consumed (Gm.)	Chlordan consumed (Gm.)
0.00	0.00 mg. / 1.00 kg.	794.5	0.00	681.0	0.00	998.8	0.00	681.0	0.00
0.05	25.50 mg. / 1.00 kg.	740.0	0.37	653.8	0.33	794.5	0.40	499.4	0.25
0.15	76.00 mg. / 1.00 kg.	653.8	0.98	399.5	0.60	399.5	0.60	281.5	0.42
0.25	125.55 mg. / 1.00 kg.	481.2	1.20	399.5	1.00	313.3	0.78	86.3	0.22
0.50	248.90 mg. / 1.00 kg.	426.8	2.33	140.7	0.70	177.1	0.89	59.0	0.30

2) Postmortem inspection of the birds fed 0.00, 0.05, and 0.15 per cent chlordan for twenty-eight days did not reveal any lesions of disease, although some of the pullets in the latter group exhibited quiescent oviducts and ovaries. The birds which received higher concentration also exhibited emaciation. Only in the 2 pullets that died, were pericarditis and distortion of the heart observed.

3) Laying pullets are highly refractory to chlordan poisoning. A question was raised relating to the possible effect of this phenomenon on the public health.

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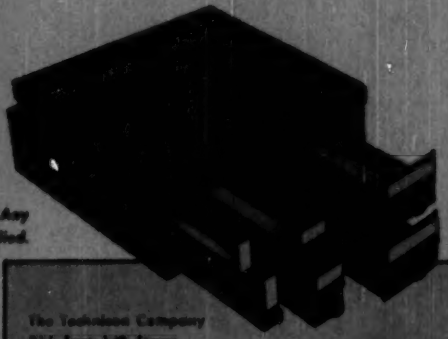

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